Gram-positive anaerobic cocci

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GRAM-POSITIVE ANAEROBIC COCCI
Identification and clinical relevance

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The printing of this thesis was supported by:
University Medical Centre Groningen, University of Groningen, BioMérieux

ISBN: 978-90-367-5047-9 (printed version)
ISBN: 978-90-367-5048-6 (electronic version)

Cover design:
Mathijs Wildeboer en Ewout Veloo

Printed by: Gildeprint Drukkerijen

RIJKSUNIVERSITEIT GRONINGEN

GRAM-POSITIVE ANAEROBIC COCCI

Identification and clinical relevance

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 7 september 2011
om 14.45 uur

door

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geboren op 27 oktober 1969
te Groningen

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Chapter 1

General introduction and scope of this thesis

General introduction

Anaerobic bacteria

Anaerobic bacteria were first observed by Louis Pasteur in 1861 [25], who described that organisms at the edge of a wet preparation became sluggish while those in the centre remained active; also, it seemed that the organisms wanted to move towards the centre. He named organisms living in the absence of oxygen “anaérobies”, from which the word anaerobes is derived. In 1877, Pasteur and Joubert [26], were able to cultivate for the first time an anaerobic bacterium, *Vibrio septique* (*Clostridium septicum*), in a liquid medium. It was isolated from an animal that died of bacteremia. After several failures to obtain growth, the idea came that this bacterium might be exclusively anaerobe. Cultivation finally succeeded under vacuum. By the 1880’s, more studies were performed on anaerobic bacteria. However, no solid media were available which hampered obtaining pure cultures. Before the death of Pasteur, in 1895, several anaerobic bacteria were isolated and described: *Clostridium butyricum*, *C. chauvoei*, *C. novyi*, *C. pasteurianum*, *C. perfringens*, *C. tetani*, and various non-sporeforming anaerobes now known as *Actinomyces israellii*, *Bacteroides fragilis*, *Fusobacterium* sp., and *Peptostreptococcus* sp. [30]. The first isolation of an anaerobic coccus was described in 1893 by Veillon [37]. The strain was isolated in pure culture from a suppurative bartholinitis. It was also noted that in three other cases the same anaerobic coccus was isolated, but in association with *Streptococcus pyogenes*.

Between 1888 and 1918, over 300 methods to obtain an anaerobic environment for culturing, were described in the literature [3]. The greatest impact on anaerobic bacteriology in routine bacteriology was the development of the anaerobic jar, which was first described by J. McIntosh and P. Fildes in 1916 [19]. Platinized charcoal and colloidal platinum were used to remove oxygen from the culture environment. This discovery led to the anaerobic jars which are nowadays used in routine bacteriology.

Anaerobic bacteria are part of the human commensal microbiota. They are part of the intestinal, oral, female genital tract and skin microbiota [10, 14]. However, they are also encountered in human infections and are isolated from approximately 30 % of all clinical samples [2]. The most encountered clinically relevant anaerobic bacteria are species from the *Bacteroides fragilis* group (43%) [2], followed by gram-positive anaerobic cocci (GPAC, 26-30%) [2, 22]. Other genera encountered are *Clostridium* sp., *Fusobacterium* sp., *Prevotella* sp., *Porphyromonas* sp., *Bilophila* sp., *Actinomyces* sp., *Bifidobacterium* sp., *Eubacterium* sp., *Eggerthella* sp., *Propionibacterium* sp. and *Veillonella* sp. In

Fig. 1 a phylogenetic tree is shown demonstrating the different genera of anaerobic bacteria which can be encountered in anaerobic infections.

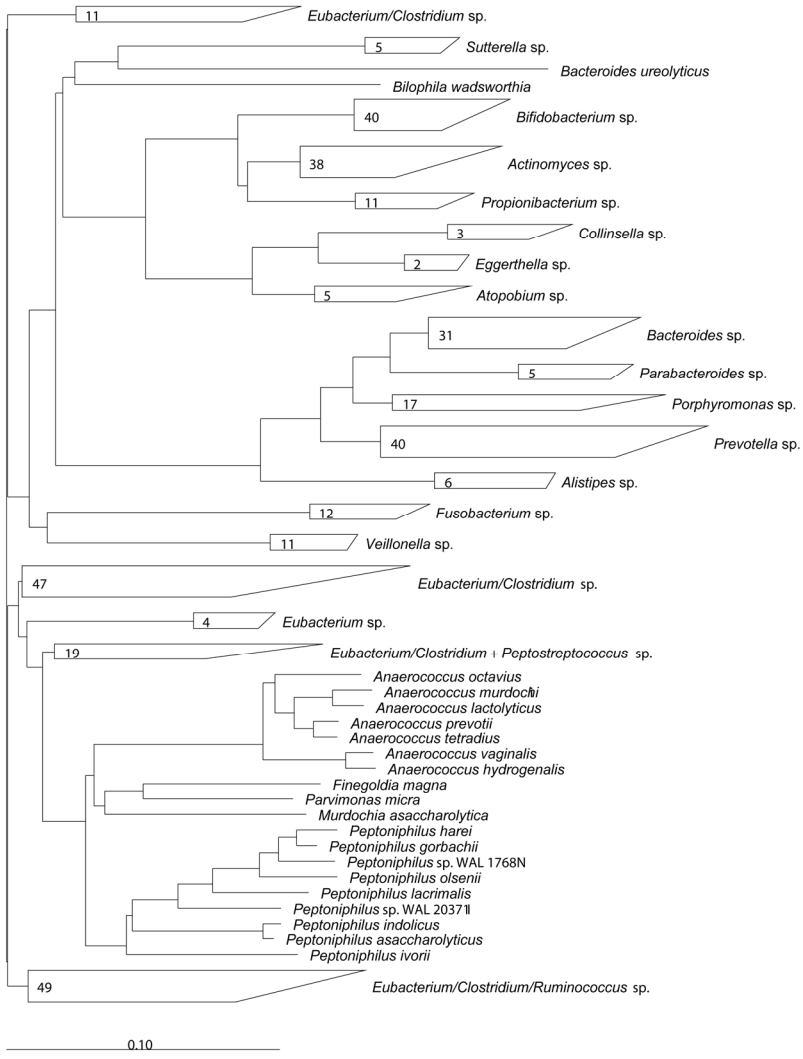

Fig. 1 A phylogenetic tree showing genera of anaerobic bacteria which can be encountered in clinical anaerobic infections. GPAC species are shown and the clusters of the genera *Peptoniphilus* and *Anaerococcus* can be observed. Including the fact that *Parvimonas*, *Finegoldia* and *Murdochia* are each a separate genus.

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Relatively much attention is being paid to *B. fragilis* sp. Reasons for that are: virulence factors and antibiotic resistance. Furthermore, *B. fragilis* sp. are more easily isolated and cultured compared to most other anaerobic bacteria. Strangely enough, the role of GPAC in anaerobic infections is often overlooked, eventhough they are the second-most often (beside *B. fragilis*) in pure culture isolated anaerobic bacteria. The disinterest in their role might be due to the fact that GPAC are in general more susceptible to antibiotics used to treat anaerobic infections than *B. fragilis* sp. In this thesis the clinical importance of GPAC, the improving methods for their identification and antibiotic resistance are discussed.

Gram-positive anaerobic cocci

GPAC were first named anaerobic streptococci, and were later generally known as peptococci or peptostreptococci. The use of molecular techniques, especially 16S rRNA gene sequencing for the identification of bacteria brought a lot of change in the taxonomy of GPAC. Analyses of 16S rRNA gene sequences revealed a large heterogeneity within the genus *Peptostreptococcus*. This resulted in a redivision of most of the *Peptostreptococcus* species. *Peptostreptococcus magnus* and *Peptostreptococcus micros* each got their own genus, *Finegoldia magna* and *Micromonas micros*, respectively [23]. Later, the name *M. micros* was shown to be illegitimate and the species was renamed as *Parvimonas* (*Pa.*) *micra* [35]. The remaining peptostreptococci were divided into three phylogenetic groups [8], *Peptoniphilus* (*Pn.*) gen. nov., *Anaerococcus* gen. nov. and *Gallicola* gen. nov., with *Gallicola barnesae* the only species present in the latter genus. The only species present in the genus *Peptostreptococcus* was *P. anaerobius*. A few years later *P. stomatis*, isolated from the human oral cavity, was added [6].

In addition to the division of species, new species, first assigned to the genus *Peptostreptococcus*, were described by Murdoch et al. [21]; *Pn. harei*, *Pn. ivorii* and *A. octavius*. Song et al. [32] described three other new species; *A. murdochii*, *Pn. gorbachii* and *Pn. olsenii*. Recently, a new genus was described, *Murdochiella* gen. nov. [36]. It contains only one species, *M. asaccharolytica*, isolated from human wound specimens.

Classical phenotypic identification

The identification of GPAC was primarily based on their strong proteolytic abilities [9]. Therefore, preformed enzymatic kits were developed in order to obtain an identification as easy and quick as possible. However, these kits were developed for the identification of most clinically relevant anaerobic bacteria and databases accompanying these kits are not always up to date. Murdoch et al. [20]

developed a scheme including only the relevant tests, which are all included in the Rapid ID 32 A system (bioMérieux), for the identification of GPAC.

Another way of fast phenotypic identification of anaerobic bacteria is the use of the ANC card for the Vitek 2 system. This card contains 36 colorimetric enzymatic tests consisting of fermentation, glycosidase and arylamidase tests, alkaline reactions and 4 other biochemical tests. As with other commercial available methods for the identification of anaerobic bacteria, only the frequently encountered anaerobes of clinical relevance are present in the database. Therefore, this method is only reliable for these anaerobes [18].

Molecular techniques

Several molecular techniques have been developed for the identification of GPAC. A PCR method for the detection of *F. magna* and *Pa. micra* in oral clinical specimens was developed by Riggio et al. [28, 29]. Furthermore, a PCR-restriction fragment length polymorphism assay of 16S rRNA genes for the identification of oral peptostreptococci was developed [27]. Yasui et al. [40, 41] developed specific DNA probes for the identification of *Pa. micra* and *P. anaerobius*. Amplification of the 16S-23S intergenic spacer region to differentiate between the different species of the former peptostreptococci was used by Hill et al. [13]. However, they were able to identify less than half of the 38 test strains, due to intra-species variation and significant differences with the type strain. Song et al. [33] developed a multiplex PCR for the identification of GPAC, using genus- and species-specific primers. In our study, species-specific 16S rRNA based probes were designed for the identification of clinically relevant GPAC [39]. To overcome the intra-species variation, the design of the probes was not only based on sequences of reference strains, but also on sequences of clinical isolates. Furthermore, the obtained identification using species-specific 16S rRNA based probes was compared with the phenotypic identification obtained using relevant enzymatic tests. The reliability of phenotypic identification for certain species of GPAC was discussed.

Our study and the study by Song et al. [33], are examples of one of the advantages of using molecular techniques for the identification of GPAC. More insight is given in the previously unknown clinical relevance of *Pn. harei*. *Pn. asaccharolyticus* and *Pn. harei* share the same biochemical features and can therefore not be phenotypically differentiated from each other [14]. *Pn. harei* plays a more important role in anaerobic infections than assumed and was in the past probably misidentified as *Pn. asaccharolyticus*. The precise role of *Pn. harei* in clinical infections still needs to be determined [38].

Novel phenotypic techniques

Recently, another technique is increasingly used for the identification of bacteria; Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). With this technique intact bacterial cells are exposed to a laser beam and the time of flight of the released proteins and protein fragments is measured. The obtained spectra are a unique fingerprint of the bacterium and allow rapid identification of different genera, species and even strains [4, 17]. Several studies have been performed in which MALDI-TOF MS has been used for the identification of anaerobic bacteria [12, 24, 34]. In each of these studies MALDI-TOF MS was shown to be superior to conventional identification methods. However, the quality and reliability of the identification depends on the quality and amount of reference spectra present in the database [12, 16, 31].

Why is it important to identify GPAC

As mentioned before, GPAC are frequently ignored in human anaerobic infections. If they are isolated from a clinical specimen, often no attempts are made to obtain a species identification and only the name peptostreptococci is given. However, there are several reasons to obtain a species identification; *i)* to detect differences in antibiotic resistance between the species, *ii)* to gain insight in the pathogenesis of different species, *iii)* to show that GPAC are associated with a specific site of infection.

F. magna is associated with bacterial vaginosis [5, 15], especially protein L expressing strains, and nonpuerperal breast abscesses [7], and is capable of producing different virulence factors. Bourgault et al. [1] showed that there is a strong association between *F. magna* found in pure culture and orthopedic procedures and post-operative wound infections. Genome analyses showed that *F. magna* exhibits beside DNA also a plasmid. There are seven sortase homologs present on the plasmid [11]. For as far as genomes are determined, this is the highest number of sortase homologs present in a gram-positive bacterium. Sortase plays an important role in the anchoring of cell-wall proteins. Since plasmids are considered to be of foreign origin, this feature contributes to the pathogenesis of *F. magna*.

Scope of this thesis

The studies presented in this thesis have the aim to give an answer to the following questions:

- Is it important to identify GPAC on the species-level?
- Which methods can be applied to obtain a reliable identification?

- Will the change in taxonomy of GPAC and/or the use of genotypic or new phenotypic methods influence our insight in the clinical relevance of certain species?
- Is there a difference in antibiotic susceptibility between the different species of GPAC, which should be taken into account in the treatment of infections, in which these distinct species are found?
- Are certain GPAC species clinically more relevant than others and are particular virulence factors present in such species which results in their presence in anaerobic infections.

Nowadays, commercial phenotypic identification methods are available for the identification of anaerobic bacteria. In Chapter 2 and 3, two of these identification methods are evaluated and the obtained phenotypic identification is compared with the genotypic identification, which is either 16S rRNA gene sequencing or fluorescent *in situ* hybridisation (FISH) using species-specific probes. In Chapter 3, the development and validation of these species-specific probes are described.

In Chapter 4, a case of a mixed aerobic/anaerobic infection is presented in which FISH was used to assist in the identification of the bacteria present.

We evaluated whether the genotypic identification of GPAC could improve the differentation between certain species and therefore give more insight in their presence and relevance in clinical material. Results of this evaluation are described in Chapter 5.

Another phenotypic method is increasingly used for the identification of bacteria, i.e. MALDI-TOF MS. In Chapter 6, the usefulness of MALDI-TOF MS for the identification of GPAC was investigated.

In Chapter 7, the antibiotic susceptibility of different species of GPAC is described.

From the literature it appears that specific virulence factors play an important role in the pathogenesis of GPAC and in particular in *F. magna*. Chapter 8 describes the different virulence factors of *F. magna* and therefore the special role of *F. magna* in certain anaerobic infections.

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Chapter 2

Evaluation of the VITEK 2 ANC card for the identification of clinical isolates of anaerobic bacteria

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J. Clin. Microbiol. 2011; 49:1745-1749

Abstract

Evaluation of the VITEK 2 ANC (bioMérieux, Marcy l'Etoile, France) card was performed with 301 anaerobic isolates. Each strain was identified by 16S rRNA gene sequencing, which is considered to be the reference method. The VITEK 2 ANC card correctly identified 239 (79.4%) of the 301 clinical isolates to the genus level including 100 species that were not claimed in the database. Correct species identification was obtained for 60.1% (181/301) of the clinical isolates. For the isolates unclaimed at the species level, correct genus identification was obtained for 47.0% (47/100) and 16 were accurately designated as not identified. Although the VITEK 2 ANC card allows rapid and acceptable identification for the most common clinically important anaerobic bacteria within 6h, improvement is required in the identification of members of the genera *Fusobacterium* sp., *Prevotella* sp., *Actinomyces* sp. and certain gram-positive anaerobic cocci (GPAC).

Introduction

The involvement of anaerobes in numerous and severe clinical infections has been reported [5, 13]. The differences in antimicrobial susceptibility [9, 16] and the development of resistance to antimicrobial drugs [16] among anaerobic bacteria have been documented. Traditional methods for identification of anaerobic pathogens are not always available in clinical bacteriology laboratories and are often laborious and time consuming [12]. Therefore, the need for a rapid and accurate method for the identification of anaerobic pathogens is highly desirable for appropriate treatment.

In the last decades, different commercial enzyme kits for the identification of clinically relevant anaerobes isolates have been developed and evaluated, e.g. the RapID-ANA II panel, the Minitek systems, the VITEK ANI card, the BBL Crystal ANR ID Kit, the API rapid ID 32 A system and the API 20 A system [6-8, 10, 14, 18, 19].

The new VITEK 2 ANC card (bioMérieux, Marcy l'Etoile, France) is designed to provide the clinical laboratory with the capability for the rapid and accurate identification of clinically relevant anaerobic bacteria and *Corynebacterium* species. The card contains 64 micro-wells with 36 colorimetric enzymatic tests. The ANC database comprises 63 taxa of anaerobic bacteria and corynebacteria. Twenty genera are listed in the Vitek 2 ANC database: *Actinomyces, Arcanobacterium, Bacteroides (Parabacteroides), Bifidobacterium, Clostridium*, *Collinsella, Corynebacterium, Eggerthella*, *Eubacterium*, *Finegoldia*, *Fusobacterium*, *Lactobacillus*, *Microbacterium*, *Parvimonas* (formerly *Micromonas*)*, Peptoniphilus*, *Peptostreptococcus, Prevotella*, *Propionibacterium*, *Staphylococcus* and *Veillonella.* The system provides only a genus level identification for

Bifidobacterium sp. and *Veillonella* sp. Of the 36 biochemical profiles, 13 are fermentation tests, 17 glycosidase and arylamidase tests, 2 alkaline reactions and 4 other biochemical tests. Additional simple off-line tests including cell morphology, gram-stain characteristics, and aerotolerance testing are required to complete the identifications.

In order to assess the accuracy of the ANC-card in a "real life" setting, unknown clinical isolates of anaerobic bacteria were used, regardless whether species are present in the VITEK database. This is in contrast to two previous studies [15, 17]. The identification obtained using the VITEK 2 ANC system was compared with that obtained using 16S rRNA gene sequencing.

Material and methods

Bacterial strains, culture conditions.

A total of 301 anaerobic clinical isolates comprising 129 gram-negative bacilli, 3 gram-negative cocci, 64 gram-positive bacilli and 105 GPAC were included in the study. All isolates were collected from patients treated at the University Medical Center Groningen, the Netherlands and were chosen randomly. Phenotypic characterization was performed with conventional methods [12]. Strains were stored at -80°C and were subcultured at least twice on *Brucella* Blood Agar (BBA, Oxoid) to ensure viability prior to inoculation in the VITEK. All culture handlings were performed in an anaerobic cabinet at 37 °C. Fast growing strains were incubated for 24h and slow growing strains for 48h–72h, before inoculation in the VITEK 2 ANC system.

Vitek analyses

Additional testing consisted of gram-character, cell morphology and aerotolerance testing. Each isolate was inoculated on a Blood Agar (BA, Oxoid) plate and incubated in 5% CO_2 atmosphere for aerotolerance testing. Inoculum suspensions were prepared in 0.45% aqueous NaCl until a turbidity between 2.70 to 3.30 McFarland was reached using a calibrated VITEK® 2 DENSICHEK (bioMérieux, Marcy l'Etoile, France).

Quality control

The quality of each batch ANC-card was determined using three control strains, including *Bacteroides vulgatus* ATCC 8482, *Clostridium perfringens* ATCC 13124 and *Corynebacterium striatum* ATCC BAA-1293. The anaerobic control strains were subcultured on BBA agar and incubated for 24h at 37°C. *C. striatum* ATCC BAA-1293 was subcultured on BA and incubated for 24 h at 37°C in 5% CO_2 atmosphere.

Sequencing

DNA of the clinical strains was extracted [4] and amplified using universal primers [11]. Obtained sequences were compared to the sequences present in GenBank using BLASTn (www.ncbi.nlm.nih.gov/blast).

Data analysis

The results in the Vitek System were classified into four categories: (i) correct identification to the species level; (ii) low discrimination, additional testing is required e.g., catalase, oxidase, β-hemolysis, pigment, motility, indole production, etc. (iii) no identification; (iv) misidentification. Species not included in the database which could not be identified by the ANC-card, were considered to be correctly classified.

Results

Clinical isolates

The set of 301 anaerobic clinical isolates contained 100 strains which were not included in the database. The overall correct species and genus identifications for the VITEK 2 ANC card were 60.1% (181/301) and 79.4% (239/301), respectively. Of the 102 misidentified isolates, 6.6% (20/301) were present in the database and 27.2% (82/301) were not.

Species present in the database

Table 1 summarizes the identification of 201 anaerobic clinical isolates which species were claimed in the database. Correct species en genus identification were 90.0% (181/201) and 95.5% (192/201), respectively, including strains identified with low discrimination but resolved by additional tests. Twenty strains (10.0%) were misidentified.

Species not present in the database

Of the species not present in the database, 84.0% (84/100) were misidentified to the species level. However, 47.0% (47/100) were correctly identified to the genus level and sixteen could not be identified using the ANC-card. These were considered to be correct results.

A total of five *Bacteroides dorei* isolates were correctly identified to the genus level. Of these, one was identified with low discrimination and four were misidentified as *Bacteroides vulgatus*. All five strains of *Bilophila wadsworthia* were misidentified as *Campylobacter ureolyticus*. Four of five isolates of *Prevotella nigrescens* and both isolates of *Fusobacterium naviforme* were misidentified as *Prevotella intermedia* and *Fusobacterium nucleatum*, respectively. Of *Peptoniphilus*

harei, 17 strains were included in the study of which sixteen were misidentified as *Peptoniphilus asaccharolyticus* and one as *Finegoldia magna*. *Clostridium citroniae* was misidentified as *F. nucleatum*. Two strains of *Clostridium hathewayi* were misidentified as *Clostridium clostridioforme*.

Table 1. Comparison of the Vitek 2 ANC card with 16S rRNA gene sequencing as a reference for the identification of 201 clinical isolates of species included in the database.

Reference ID (n)	No. (%) of isolates				
	Correct species ID	Correct genus ID	Low discri-mination	No ID	Incorrect species ID
Gram-negative strains (95)					
***Bacteroides fragilis* sp. (76)**	**68 (89.5)**	**75 (98.7)**	**1**	**0**	**9**
B. fragilis (42)	38 (90.5)	41 (97.6)	0	0	4
B. uniformis (4)	2	4	0	0	2
B. ovatus (8)	8	8	0	0	0
B. vulgatus (5)	5	5	0	0	0
B. thetaiotaomicron (16)	14 (87.5)	16 (100.0)	1	0	3
B. caccae (1)	1	1	0	0	0
***Parabacteroides* sp. (4)**	**3**	**3**	**0**	**1**	**1**
P. distasonis (4)	3	3	0	1	1
***Campylobacter* sp. (2)**	**1**	**1**	**0**	**1**	**1**
C. ureolyticus (2)	1	1	0	1	1
***Prevotella* sp. (6)**	**5**	**6**	**0**	**0**	**1**
P. buccae (2)	1	2	0	0	1
P. disiens(1)	1	1	0	0	0
P. bivia (1)	1	1	0	0	0
P. melaninogenica (2)	2	2	0	0	0
***Fusobacterium* sp. (7)**	**5**	**7**	**1**	**0**	**2**
F. nucleatum (4)	4	4	0	0	0
F. necrophorum (2)	1	2	1	0	1
Fusobacterium sp. (1)	0	1	0	0	1
Gram-positive strains (106)					
***GPAC* (55)**	**54 (98.2)**	**54 (98.2)**	**1**	**1**	**1**
Finegoldia magna (33)	33 (100.0)	33 (100.0)	0	0	0
Parvimonas micra (18)	17 (94.4)	17 (94.4)	1	1	1
Peptostreptococcus anaerobius (4)	4	4	0	0	0
***Propionibacterium* sp. (16)**	**13 (81.3)**	**13 (81.3)**	**3**	**0**	**3**
P. acnes (15)	13 (86.7)	13 (86.7)	3	0	2
P. granulosum (1)	0	0	0	0	1

Table 1. Continued

Reference ID (n)	No. (%) of isolates				
	Correct species ID	Correct genus ID	Low discri-mination	No ID	Incorrect species ID
***Actinomyces* sp. (5)**	**3**	**3**	**0**	**1**	**2**
A. meyeri (1)	1	1	0	0	0
A. israellii (3)	2	2	0	1	1
A. naeslundii (1)	0	0	0	0	1
***Clostridium* sp. (27)**	**27 (100.0)**	**27 (100.0)**	**7**	**0**	**0**
C. difficile (15)	15 (100.0)	15 (100.0)	3	0	0
C. perfringens (7)	7	7	0	0	0
C. septicum (1)	1	1	0	0	0
C. baratii (1)	1	1	1	0	0
C. butyricum (3)	3	3	3	0	0
***Eggerthella* sp. (2)**	**2**	**2**	**0**	**0**	**0**
E. lenta (2)	2	2	0	0	0
***Bifidobacterium* sp. (1)**	**0**	**1**	**0**	**0**	**0**

GPAC, Gram-positive anaerobic cocci

Discussion

In this study, we assessed the reliability of the VITEK ANC card system for identification of anaerobic bacteria isolated from clinical materials. *B. dorei* isolates, not included in the Vitek 2 ANC database, were misidentified as *B. vulgatus*. The enzymatic activities of the VITEK 2 ANC card showed that all *B. dorei* and *B. vulgatus* were esculin-positive and esculin-negative, respectively. However, this biochemical reaction was in contrary to the report of Bakir et al. [2], who described that *B. dorei* is esculin-negative. If the species *B. dorei* had been included in the database, the VITEK 2 ANC card could have distinguished between the two species by interpreting the esculin hydrolysis reaction. So far the full potential of the VITEK 2 ANC card is not being used. Validating more strains is required to determine whether this feature can be used to distinguish *B. dorei* from *B. vulgatus*.

B. wadsworthia, not present in the database, *was* consistently misidentified as *C. ureolyticus*. The VITEK 2 ANC card could have differentiated *B. wadsworthia* from *C. ureolyticus*. The biochemical profiles obtained by the VITEK 2 ANC system revealed that *C. ureolyticus* was positive for urease and leucine arylamidase, while *B. wadsworthia* was urease positive and leucine arylamidase negative. The positive leucine arylamidase reaction separates *C. ureolyticus* from *B. wadsworthia* [1]. We recommend that *B. wadsworthia* can be included in the database and this should be validated in practice.

Another species not included in the database, *C. clostridioforme* cannot be differentiated from *C. hathewayi* by using the VITEK 2 ANC card. Therefore, we

Table 2. Comparison of the VITEK 2 ANC card with the 16S rRNA gene sequencing as reference identification for the identification of 100 clinical isolates which species are not included in the database.

Reference ID	No (%) of isolates				
	Correct species ID	Correct genus ID	Low discri-mination	No ID	Incorrect species ID
Gram-negative strains (37)					
***Bacteroides fragilis* sp. (9)**	**0**	**7**	**1**	**0**	**9**
Bacteroides sp. (1)	0	1	0	0	1
B. suis (1)	0	0	0	0	1
B. tectus (1)	0	0	0	0	1
B. salyersiae (1)	0	1	0	0	1
B. dorei (5)	0	5	1	0	5
***Odoribacter* sp. (1)**	**0**	**0**	**0**	**1**	**0**
O. splanchnicus (1)	0	0	0	1	0
***Parabacteroides* sp. (2)**	**0**	**0**	**0**	**1**	**1**
P. goldsteinii (1)	0	0	0	0	1
P. merdae (1)	0	0	0	1	0
***Bilophila* sp. (5)**	**0**	**0**	**0**	**0**	**5**
B. wadsworthia (5)	0	0	0	0	5
***Prevotella* sp. (8)**	**0**	**8**	**0**	**0**	**8**
P. nigrescens (5)	0	5	0	0	5
P. nanceiensis (1)	0	1	0	0	1
P. oris (1)	0	1	0	0	1
P. denticola (1)	0	1	0	0	1
***Fusobacterium* sp. (2)**	**0**	**2**	**0**	**0**	**2**
F. naviforme (2)	0	2	0	0	2
***Porphyromonas* sp. (2)**	**0**	**0**	**0**	**1**	**1**
P. somerae (1)	0	0	0	0	1
P. catoniae (1)	0	0	0	1	0
***Campylobacter* sp. (3)**	**0**	**0**	**1**	**1**	**2**
C. rectus (2)	0	0	0	1	1
C. gracilis (1)	0	0	1	0	1
***Dialister* sp. (1)**	**0**	**0**	**0**	**0**	**1**
D. pneumosintes (1)	0	0	0	0	1
***Sutterella* sp. (1)**	**0**	**0**	**0**	**1**	**0**
S. stercoricanis (1)	0	0	0	1	0
***Veillonella* sp. (3)**	**0**	**2**	**1**	**0**	**3**
V. parvula (2)	0	2	0	0	2
V. atypica (1)	0	0	1	0	1

Table 2. Continued.

Reference ID	No (%) of isolates				
	Correct species ID	Correct genus ID	Low discri-mination	No ID	Incorrect species ID
Gram-positive strains (63)					
GPAC (49)	**0**	**18 (36.7)**	**7**	**11(22.4)**	**38 (77.6)**
Peptoniphilus octavius (1)	0	0	0	1	0
Peptostreptococcus stomatis (1)	0	0	0	0	1
Peptoniphilus ivorii (3)	0	0	1	0	3
Peptoniphilus lacrimalis (2)	0	1	1	0	2
Peptoniphilus harei (17)	0	16 (94.1)	0	0	17 (100.0)
Peptoniphilus gorbachii (2)	0	1	1	0	2
Peptococcus niger (1)	0	0	0	0	1
Anaerococcus sp. (3)	0	0	1	1	2
Anaerococcus murdochii (4)	0	0	1	3	1
Anaerococcus vaginalis (6)	0	0	0	2	4
Anaerococcus prevotii (2)	0	0	0	2	0
Anaerococcus tetradius (2)	0	0	0	2	0
Atopobium parvulum (2)	0	0	0	0	2
Atopobium rimae (3)	0	0	2	0	3
***Actinomyces* sp. (5)**	**0**	**4**	**0**	**0**	**5**
A. georgiae (1)	0	1	0	0	1
A. odontolyticus (2)	0	1	0	0	2
A. graevenitzii (1)	0	1	0	0	1
A. gerencseriae (1)	0	1	0	0	1
***Clostridium* sp. (7)**	**0**	**6**	**1**	**0**	**7**
C. citroniae (1)	0	0	0	0	1
C. innocuum (1)	0	1	1	0	1
C. hathewayi (2)	0	2	0	0	2
C. scindens (1)	0	1	0	0	1
C. disporicum (1)	0	1	0	0	1
C. coccoides (1)	0	1	0	0	1
***Lactobacillus* sp. (1)**	**0**	**0**	**1**	**0**	**1**
L. catenaformis (1)	0	0	1	0	1
***Robinsoniella* sp. (1)**	0	0	0	0	1
R. peoriensis (1)	0	0	0	0	1

recommend that the VITEK2 ANC system should give identification with low-level discrimination involving both species mentioning that additional biochemical features should be determined [20]. In our study, *C. citroniae* was incorrectly identified as *F. nucleatum*. This incorrect identification was due to the gram-

character which was used in the off-line tests. Certain clostridia, e.g. *C. clostridioforme*, *C. hathewayi* and *C. citroniae* tend to stain gram-negative. Susceptibility to special potency antibiotic disks of vancomycin, kanamycin and colistin is recommended to aid in determining the gram-character [12].

As expected, the VITEK system cannot differentiate between species which are difficult to distinguish from each other phenotypically, e.g. *P. nigrescens* from *P. intermedia*, *F. naviforme* from *F. nucleatum* and *P. harei* from *P. asaccharolyticus* [12]. Therefore, the result should be given as low-level discrimination by including both species instead of one. If possible with mentioning additional testing, e.g. cell morphology differentiation for *F. naviforme* and *F. nucleatum*. Cells of *F. nucleatum* are slender with pointed ends, while cells of *F. naviforme* are boat-shaped [12].

Generally, the ANC card is unreliable for identifying species belonging to the genus *Actinomyces* and certain GPAC species (Table 2). For those isolates included in the database, a reliable identification was obtained for the genera *Bacteroides fragilis*, *Clostridium*, *Prevotella*, *Fusobacterium*, *Parvimonas*, *Finegoldia*, *Peptostreptococcus* and the species *Eggerthella lenta*.

In a recent study of Blairon et al. [3], 196 clinical isolates were tested, some of which were not included in the VITEK ANC database. They reported a correct species and genus identification for 51.5% and 70.9%, respectively. In our study, the VITEK ANC card provided better results 60.1% and 79.4% correct species and genus identifications respectively. This may be explained by the fact that their collection of strains was not based on sequenced species. They compared the identification obtained with the VITEK 2 ANC card with those obtained with the Microbial Identification System (MIS) complemented with necessary biochemical tests. Secondly, species not included in the database which were correctly classified as no identification were not assigned as a correct result. Therefore, their interpretation result cannot fully be compared to our study.

In contrast to other validations of the ANC card, Mory et al. [15] and Rennie et al. [17] reported a correct species identification of 86.5% and 95.1%, respectively. However, species and genera not present in the database had been eliminated from their study isolates [15, 17]. The lower level of correct identification obtained in our clinical study can be explained by the inclusion of test isolates which are not present in the database, but are encountered in clinical material. With our recommendations, the correct species and genus identification would increase from 60.1% (181/301) and 79.4% (239/301) to 70.1% (211/301) and 81.1% (244/301), respectively. These percentages of correct identification are still lower than those from the previously published studies [15, 17]. However, including their 169 excluded isolates [17], which were unclaimed by the system, a correct genus

and species identification would be obtained of 90.0% and 67.0%, respectively. These numbers are comparable to those from our study.

High inoculum density is required for the inoculation of the VITEK 2 ANC card. Fast growing bacteria are good candidates for VITEK 2 ANC identification. However, fastidious anaerobes such as *C. ureolyticus* and *B. wadsworthia* require several agar plates to obtain a sufficiently large inoculum. Cell morphology and gram-characteristics provide useful information to avoid misidentification of the micro-organism. Especially the gram-character of gram-negative staining isolates should be confirmed using the special potency disks [12].

The VITEK 2 ANC card provides a reliable identification for a limited number of relevant anaerobic bacterial species in a routine diagnostic setting. The system performs inadequately concerning species not present in the database. For certain species not included in the database (Table 2), it would benefit from limiting the identification to the genus level. Improvement and extension of the database may result in a more accurate identification.

Acknowledgements

The author would like to acknowledge Willy Baas and Wietske Postma for molecular technical support.

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Chapter 3

Development of 16S rRNA-based probes for the identification of gram-positive anaerobic cocci isolated from human clinical specimens

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Clin. Microbiol. Infect. 2007; 13:985-992.

Abstract

Fluorescent probes targeted at the 16S rRNA of most clinically encountered gram-positive anaerobic cocci (GPAC) were designed and applied on clinical isolates. Probes were developed for *Peptostreptococcus anaerobius* and *P. stomatis* (Pana134), *Parvimonas micra* (Pamic1435), *Finegoldia magna* (Fmag1250), *Peptoniphilus asaccharolyticus* (Pnasa1254), *Pn. ivorii* (Pnivo731), *Pn. harei* (Pnhar1466), *Anaerococcus vaginalis* (Avag1280) and *A. lactolyticus* (Alac1438). Probes were designed using 16S rRNA sequences of reference strains and 88 randomly chosen clinical isolates. These strains were also used for validation of the probes. An additional group of 100 clinical isolates was used to determine how many GPAC strains could be identified with this set of probes. Of these strains 87 % could be identified. Clinical isolates (n=13) which could not be identified were sequenced. Most of these strains were other GPAC not targeted by the probes. No clinical isolates of *Pn. asaccharolyticus* were encountered. Near full-length sequences were obtained from 77 out of 101 (n=88 + 13) sequenced clinical isolates. Of these, 25 showed <98 % similarity with their closest established species. The Fmag1250, Pamic1435, Pnhar1466, Pana134, Pnasa1254 and Pnivo731 probes gave a reliable identification and hybridized with all corresponding strains. The Avag1280 and Alac1438 probes failed to hybridize with some of the corresponding strains 2 and 1 strain, respectively, due to intra-species variation. Using the described set of probes a fast and reliable identification of clinical isolates was obtained.

Introduction

Gram-positive anaerobic cocci (GPAC) are part of the commensal microbiota of humans at different sites of the body, but are also known to be important in human disease. They account for about one third of the anaerobic isolates recovered from clinical material [22]. During the last decade, the taxonomy of GPAC has changed dramatically [4, 16, 21]. *Peptostreptococcus productus* (*P.*) was added to the genus *Ruminococcus* [7] and the genus *Peptostreptococcus* was divided into six new groups [6, 23]. Murdoch et al. [23] transferred the species *P. micros* and *P. magnus* to two new genera, *Micromonas* and *Finegoldia*, respectively, each the only species present in the genus. Recently the genus *Micromonas* was replaced by *Parvimonas* (*Pa.*), with *Pa. micra* (formerly *M. micros*) the only species present [29]. Ezaki et al. [6] divided the remaining peptostreptococci into three different phylogenetic groups, *Peptoniphilus* (*Pn.*) gen. nov, *Anaerococcus* gen. nov. and *Gallicola* gen. nov, with *G. barnesae* as the only species present in the latter genus. The type species of the other two new genera being *Pn. asaccharolyticus* and *A. prevotii*, respectively. The only species left in the

genus of *Peptostreptococcus* are *P. anaerobius*, and a recently described species *P. stomatis* isolated from the human oral cavity [5].

Infections involving GPAC are often polymicrobial [9]. The most commonly encountered GPAC in infections are *P. anaerobius*, *Pa. micra*, *F. magna* and *Pn. asaccharolyticus* [32]. Although these anaerobic cocci appear commonly in infections, little attention is paid to their detection and identification in diagnostic laboratories due to cumbersome and inadequate classification systems. The phenotypic identification of GPAC is based on morphological appearance, carbohydrate formation and gas liquid chromatography (GLC) [15]. The introduction of proteolytic enzyme profiles made the identification of the acknowledged GPAC easier and reproducible [8]. This contributed to the development of several commercial enzyme kits, such as RapID ANA (Innovative Diagnostic Systems, Atlanta, Ga.) and Rapid ID 32A (API-bioMerieux,Basingstoke, United Kingdom). However, newly described species are not added to the database of these kits. Several new species, originally assigned to the genus of *Peptostreptococcus* have been described, e.g. *Pn. harei*, *Pn. ivorii* and *A. octavius* [20]. The clinical relevance of these new species has not yet been assessed.

The availability of genotypic data makes it possible to develop molecular techniques for the detection and identification of GPAC. The usefulness of 16S rRNA sequences for identification of GPAC was evaluated by Song *et al.* [27]. These authors were able to identify 84 % of the clinical isolates of GPAC, using their own sequence data base of type strains. These sequences revealed ambiguous data in public databases. Song *et al.* [28] developed a multiplex PCR assay for the rapid identification of GPAC, using genus- and species-specific primers. Other molecular detection assays for GPAC were developed by Riggio *et al.* [24, 25, 26], including a PCR method for the detection of *F. magna* and *Pa. micra* in oral clinical specimens and a PCR-restriction fragment length polymorphism assay of 16S rRNA genes for the identification of oral *Peptostreptococcus* isolates. DNA probes have also been used for the identification of *Pa. micra* [33] and *P. anaerobius* [34]. The amplification of the 16S-23S intergenic spacer region (ISR) was used by Hill *et al.* [12] to differentiate between different species of the former peptostreptococci. When the banding patterns of 38 test strains were compared with banding patterns of reference strains, less than half the strains could be identified. The other strains could not be identified due to intra-species variation or they differed significantly from the type strain.

GPAC were also detected in fecal samples using PCR [31] and quantified using fluorescent *in situ* hybridization (FISH) [11]. Especially the latter technique makes it possible to detect and identify GPAC in pure culture and probably also

directly in clinical specimens. This technique is easy to perform, cheap compared with other molecular methods and can easily be implemented in diagnostic laboratories. Here we describe the design and validation of 16S rRNA based probes for the detection and identification of clinically relevant GPAC. Probes were designed for a selection of GPAC isolated in our laboratory, *viz. P. anaerobius*/*stomatis*, *Pa. micra*, *F. magna, Pn. asaccharolyticus, A. vaginalis*, *A. lactolyticus*, *Pn. ivorii* and *Pn. harei*.

Material and methods

Strains

Reference strains and clinical isolates were cultured on Brucella Blood agar (BBA) anaerobic plates and incubated in an anaerobic atmosphere for 48 hours at 37 ºC. Cells were harvested and fixed for FISH analyses in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, per liter) and 96 % ethanol (1:1, v/v). Fixed cells were stored at -20 ºC. A total of 188 clinical isolates was randomly obtained from the diagnostic laboratory of our department and the Regional Public Health Laboratory in Enschede. The clinical materials were obtained from a variety of sites, e.g. abdomen, the head/neck area, leg, arm, groin. From each patient only one isolate from each species was used. The clinical isolates of GPAC were phenotypically identified using Rapid ID 32A and the Wadsworth manual [15]. Isolates which could not be identified, or when an ambiguous identification was obtained, were designated as GPAC. Phenotypically, *Pn. asaccharolyticus* can only be distinguished from *Pn. harei* by colony form and cell morphology [15]. Since this is difficult to achieve, they were identified as *Pn. asaccharolyticus*/ *Pn. harei*.

16S rRNA gene sequencing

The 16S rRNA gene of 88 randomly chosen clinical isolates were sequenced. DNA of the strains was isolated as described previously [3] and the 16S rRNA gene was amplified and sequenced using universal 16S rRNA-specific primers [13].

Sequence data analysis

The sequences were aligned against sequences of reference strains derived from the European Molecular Biology Laboratory (EMBL) database and the Ribosomal Database Project (RDP) [18], using the ARB-software [17]. Similarities of the sequences of the clinical isolates with those of their closest established species were calculated using a DNA distance matrix embedded in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The *Escherichia coli* positions 82

and 1371 were included, to ensure accurate determination of species similarity. A phylogenetic tree of sequences with <98 % similarity with their closest relative, was constructed using the same alignment by the neighbour joining method with Jukes Cantor correction based on a distance matrix including only positions with >50 % conservation and parsimony, implemented in the ARB- software. As a control the phylogenetic tree was also calculated using the Maximum Parsimony and Maximum Likelihood methods. The topology of the tree was calculated with bootstrap analyses of 1000 replicate trees. Sequences with <98 % similarity were deposited in the EMBL database and accession numbers were assigned (Fig. 1).

Probe design

Probes (Table 1) were designed using the sequences of clinical isolates and reference strains present in the RDP [18] and EMBL database. ARB-software was used for aligning and probe design [17]. When necessary, unlabeled helper nucleotides (Table 2) were designed to increase the *in situ* accessibility of 16S rRNA, as described by Fuchs et al. [10]. For practical purposes, probes were designed to have a similar hybridization temperature. All probes were labeled with fluorescein-5-isothiocyanate (FITC) at the 3'and 5'end and synthesized commercially (Eurogentec, Seraing, Belgium).

Validation

Cells of ethanol-fixed strains were spotted on a slide and fixed for 10 min using 96 % ethanol (v/v). Hybridizations were performed at 50 ºC in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.1% SDS [wt/vol]) containing 10 ng probe, as described previously [14]. For the Pana134, Pivo731 and Avag1280 probe, 5 ng of the appropriate helper nucleotides was added to the hybridization mixture. Hybridizations were performed overnight. The bacterial probe EUB338 [2] served as a positive control and its complement non-EUB338 [30] as the negative control. The specificity of the Pana134 and Pnasa1254 probes was increased by adding 20 % formamide (v/v), a duplex destabilizing agent, to the hybridization buffer. The hybridization signal obtained with the species-specific probes was compared visually with the EUB338 and non-EUB338 signal, using an epifluorescence Olympus BH2 microscope (Hamburg, Germany), and scored negative or positive. Strains of *F. magna*, *A. vaginalis* and *Pn. ivorii* were permeabilized with 500 µg/ ml (50 mM Tris-HCl [pH 7.6]) proteinase-K solution. The spotted cells were covered with the proteinase-K solution and incubated for 10 min at room temperature. The enzymatic reaction was stopped by incubating the slides in 96 % ethanol (v/v) for 2 min. All probes were validated against reference strains listed in Table 3, and the sequenced clinical isolates. To assess how many of the GPAC can be identified,

the set of species-specific probes was also applied to an additional group of 100 clinical isolates.

Table 1. Species-specific probes for the detection and identification of GPAC and sequence alignment of the target sites.

Probe	Reference strain[a]	Target-site
Pamic1435 (S-S-P.mic-1435-b-A-18)[b]		5'**TGCGGTTAGATCGGCGGC** 3'
	***Pa. micra* DSM 20468^T**	3'ACGCCAAUCUAGCCGCCG 5'
	Desulfovibrio putealis DSM 16056^T	----------GG-------
	Leptotrichia hofstadii CCUG 47504^T	----------G----U--
Fmag1250 (S-S-F.mag-1250-b-A-18)		5'**CACTATATAGCTKCCCTC**[c] 3'
	***F. magna* DSM 20470^T**	GUGAUAUAUCGAMGGGAG[d]
	Chryseobacterium meningosepticum AMA	-----CC----------
	gpac218[e]	----C-G----------
Pnasa1254 (S-S-P.asa-1254-a-A-18)		5'**CTATCACTAGCTCGCCCG** 3'
	***Pn. asaccharolyticus* DSM 20463^T**	GAUAGUGAUCGAGCGGGC
	Pn. indolicus DSM 20464^T	-----C------A-----
Avag1280 (S-S-A.vag-1280-a-A-18)		5'**GGGACAGACTTTGTGAGT** 3'
	***A. vaginalis* DSM 7457^T**	CCCUGUCUGAAACACUCA
	A. hydrogenalis GIFU 7662	--------A---------
	gpac047	--------A---------
	A. prevotii DSM 20548^T	-------C----A-----
	Clostridium alcaliphilum B8NS1-A	---------------C-C
	Proteus mirabilis U1-2	UG----------U-----
	C. aminovorans B7FT-A	-----G---------C-C
	A. lactolyticus DSM 7456^T	-------C----A----U
Alac1438 (S-S-A.lac-1438-a-A-18)		5'**CCACAAGGGTTCGCTCAC** 3'
	***A. lactolyticus* DSM 7456^T**	GGUGUUCCCAAGCGAGUG
	Bacillus thermocloaceae DSM 5250^T	---U--------------
	gpac053	---A--U-----------
	Marinococcus halophilus lv4	---C--------G-----
	Enterococcus moraviensis DSM 15919^T	----------UG-----
	Thermohalobacter berrensis CNCM105955^T	---U-------U------
	A. vaginalis DSM 7457^T	--AU--U-----------
Pnivo731 (S-S-P.ivo-0731-a-A-18)		5'**TTGAAGTCCAGCAAGTCG** 3'
	***Pn. ivorii* DSM 10022^T**	AACUUCAGGTCGUUCAGC
	A. prevotii JCM 8142	--N--------A------[f]
	F. magna 1-68	--A--------A------

Table 1. Continued

Probe	Reference strain[a]	Target-site
	C. sticklandii VPI 14603	---A-------U------
	C. ultunense DSM 10521^T	-A---------U------
	Filifactor alocis ATCC 35896^T	---G-------U------
	Leuconostoc fallax DSM 20189^T	-G------------GU--
	P. anaerobius DSM 2949^T	---G-------U---G--
	C. beijerinckii DSM 791^T	--UG-------U------
Pnhar1466 (S-S-P.har-1466-a-A-18)		5'**GTCACYTATCCTACCTTC**[g] 3'
	***Pn. harei* DSM 10020^T**	CAGUGRAUAGGAUGGAAG[h]
	F. magna DSM 20470^T	----A-U-----------
	C. sporosphaeroides DSM 1294^T	---C--U-----------
	Eubacterium cylindroides DSM 3983^T	----A-C-----------
	Streptococcus pleomorphus DSM 20574^T	---NA-C-----------
	C. akagii DSM 12554^T	U-----C-----------
	Fischerella muscicola ATCC 29114	------UC----------
	Olisthodiscus luteus	------U----------U
	gpac032	----A-U-----------
	gpac127	-----A-C----------
	C. novyi ATCC19402	U-----U----G------
	Dermabacter hominis	U-----C----G------
Pana134 (S-S-P.ana-0134-a-A-22)		5'**ATGTTATCCATGTGTATAGGGC** 3'
	P. anaerobius DSM2949^T	UACAAUAGGUACACAUAUCCCG
	P. stomatis DSM17678^T	----------
	C. colinum DSM6011^T	---------G----------
	E. tenue DSM20695^T	---------C------G-----
	C. piliforme	---------GU----------
	C. glycolicum DSM1288^T	----------CU---
	C. paradoxum DSM7308^T	C---------U-----G-----
	C. difficile 79685	---------C------G----A

[a] Standardized nomenclature [27].

[b] Only strains with relevant mismatches are shown. The target-species and the probe are indicated in bold.

[c] K, an G/T nucleotide degeneracy.

[d] M, an C/A nucleotide degeneracy.

[e] Clinical isolate sequenced in this study.

[f] N, an G/T/C/A nucleotide degeneracy.

[g] Y, an C/T nucleotide degeneracy.

[h] R, an G/A nucleotide degeneracy.

Table 2. Unlabeled helper nucleotides used in combination with species-specific probes to increase the *in situ* accessibility of the 16S rRNA.

Probe	Helpernucleotide	Sequence (5'-3')
Avag1280	H1263	TTCGCTTCAGATCGCTCT
	H1299	GCAGAGTACAATCCGAACT
Pnivo731	H716	CCTTCGCCACCGGTAT
	H750	CGTTCCTCAGCGTCAG
Pana134	H115	AGGTTACCCACGCGTTACT
	H155	TATTATCATGTATTAGTAAACTTTTCAGT

Results

The designed set of probes demonstrated 100 % specificity when validated against reference strains (Table 3). *F. magna*, *A. vaginalis* and *Pn. ivorii* needed permeabilization with proteinase-K prior to hybridization. All other strains could be hybridized without additional treatment. The set of probes was also validated against sequenced clinical isolates (Table 4). All probes hybridized only with their corresponding clinical isolates.

The set of probes was applied to an additional group of 100 clinical isolates and the genotypic results compared with the phenotypic identification. Since the phenotypic identification of GPAC is hindered by inadequate identification schemes, the results are classified by their genotypic identification obtained with the 16S rRNA-based probes (Table 5). The majority of the strains were identified as being *F. magna* (29 %), genotypically and phenotypically. The Pamic1435 probe hybridized with 28 % of the clinical isolates. Three of these strains were phenotypically misidentified as *F. magna* and one could not be identified.

The Pana134 probe hybridized with 4 % of the clinical isolates, all phenotypically identified as *P. anaerobius* strains. The weak hybridization signal of this probe was increased by adding the unlabeled helper nucleotides H115 and H155 (Table 2). The Pnhar1466 probe hybridized with 17 % of all clinical isolates. Four of these strains were phenotypically misidentified, one as *Pn. indolicus* and the other three could not be identified using the current identification scheme. Of all strains, 6 % were genotypically identified as *Pn. ivorii* with four strains correctly phenotyped and two strains phenotyped incorrectly. The Avag1280 probe hybridized with 2 of the clinical isolates (2 %) and agreed with their phenotypic identification. The hybridization signal of the Pnivo731 and Avag1280 probe was increased using the helper nucleotides H716 + H750 and H1263 + H1299,

Table 3. Validation of the species-specific probes for GPAC against reference strains and clinical isolates.

Strain	Probe							
	Fmag 1250	Mmic 1435	Pnhar 1466	Pana 134[a,b]	Avag 1280[b]	Alac 1438	Pnivo 731[b]	Pnasa 1254[a]
F. magna DSM20470[T, c, d]	+	-	-	-	-	-	-	-
Pa. micra DSM20468[T]	-	+	-	-	-	-	-	-
Pn. harei DSM10020[T]	-	-	+	-	-	-	-	-
P. anaerobius DSM2949[T]	-	-	-	+	-	-	-	-
A. vaginalis DSM7457[T, d]	-	-	-	-	+	-	-	-
A. lactolyticus DSM7456[T]	-	-	-	-	-	+	-	-
Pn. ivorii DSM10022[T, d]	-	-	-	-	-	-	+	-
Pn.asaccharolyticus DSM20463[T]	-	-	-	-	-	-	-	+
A. prevotii DSM20548[T]	-	-	-	-	-	-	-	-
A. tetradius MMB[e]	-	-	-	-	-	-	-	-
Pn. indolicus DSM20464[T]	-	-	-	-	-	-	-	-
gpac218[f]	-	-	-	-	-	-	-	-
gpac053	-	-	-	-	+	-	-	-
gpac032	+	-	-	-	-	-	-	-
gpac127	-	-	-	-	-	-	-	-
C.sporosphaeroides DSM1294[T]	nd[g]	nd	-	nd	nd	nd	nd	nd
Dermabacter hominis DSM7083[T]	nd	nd	-	nd	nd	nd	nd	nd
C. colinum DSM6011[T]	nd	nd	nd	-	nd	nd	nd	nd
E. tenue DSM20695[T]	nd	nd	nd	-	nd	nd	nd	nd
C. glycolicum DSM1288[T]	nd	nd	nd	-	nd	nd	nd	nd
C. difficile DSM1296[T]	nd	nd	nd	-	nd	nd	nd	nd
Proteus mirabilis MMB	nd	nd	nd	nd	-	nd	nd	nd
C. beijerinckii MMB	nd	nd	nd	nd	nd	nd	-	nd
Enterococcus moraviensis DSM15919[T]	nd	nd	nd	nd	nd	-	nd	nd

[a] To increase specificity of the hybridization, 20 % formamide was added to the hybridization buffer.

[b] Unlabeled helper nucleotides were added to the hybridization mixture to increase the *in situ* accessibility of the 16S rRNA.

[c] Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

[d] Prior to hybridization cells were permeabilized with 500 µg/ml proteinase-K for 10 min at room temperature.

[e] All MMB strains are clinical isolates and have been identified by routine procedures.

[f] All gpac strains are sequenced clinical isolates used in this study.

[g] Not determined.

Table 4. Validation of the newly designed probes against 88 clinical isolates of which the sequence of the 16S rRNA was used for the design of the probes.

	Identification (n)	
Species name	Sequence	Probe
F. magna	26	26
Pa. micra	13	13
Pn. harei	16	16
A. lactolyticus	7	7
A. vaginalis	6	6
Pn. ivorii	5	5
P. anaerobius	5	5
Pn. lacrimalis	3	
A. tetradius	1	
A. hydrogenalis	1	
Peptococcus niger	1	
Atopobium parvulum	2	
Ruminococcus gnavus	1	
Peptostreptococcus sp. E3_32	1	

respectively (Table 2). The Alac1438 probe hybridized with 1 of the clinical isolates (1%) that could not be phenotypically identified.

With this set of probes 87 % of all isolated GPAC could be identified. The remaining 13 strains were sequenced to determine whether the new probes had hybridized correctly. The sequence results show a variety of GPAC. Two strains were identified as *Peptococcus niger* (*Pe.*), two as *A. vaginalis*, one as *A. lactolyticus* and one as *Sarcina ventriculi*. Of these six strains, one of the *A. vaginalis* strains was phenotypically misidentified as *Pn. lacrimalis* and the *A. lactolyticus* was misidentified as *F. magna*. The other 4 strains were phenotypically correctly identified. Seven strains could not be phenotypically identified. With sequence analysis they were identified as *Atopobium parvulum* (*At.*) (3 strains), *A. tetradius* (1 strain), *Pn. octavius* (1 strain) and *Bacterium* N14-24 (1 strain). One strain could not be identified because amplification of the 16S rRNA gene was unsuccessful. Sequence analysis of the *A. vaginalis* strains and *A. lactolyticus* strain revealed that the corresponding probes were negative for these strains due to mismatches. The *A. lactolyticus* had 1 mismatch with the Alac1438 probe and the two *A. vaginalis* strains had 1 and 3 mismatches, respectively.

A distance matrix was calculated from all sequence results which yielded a near complete full-length sequence. Partially determined sequences were suitable for probe design but not for phylogenetic analysis. Near full length sequence were

Table 5. Application of the species-specific probes on an additional group of 100 clinical isolates.

Probe ID (n)	Phenotypic ID (n)	Sequence ID (n)
F. magna (29)	*F. magna* (29)	
Pa. micra (28)	*Pa. micra* (24)	
	F. magna (3)	
	GPAC (1)	
Pn. harei (17)	*Pn. asaccharolyticus*/ *Pn. harei* (13)	
	Pn. indolicus (1)	
	GPAC (3)	
A. lactolyticus (1)	GPAC (1)	
A. vaginalis (2)	*A. vaginalis* (2)	
Pn. ivorii (6)	*Pn. ivorii* (4)	
	GPAC (2)	
P. anaerobius (4)	*P. anaerobius* (4)	
no ID (13)	*Pe. niger* (2)	*Pe. niger* (2)
	A. vaginalis (1)	*A. vaginalis* (1)
	Pn. lacrimalis (1)	*A. vaginalis* (1)
	F. magna (1)	*A. lactolyticus* (1)
	Sarcina ventriculi (1)	*S. ventriculi* (1)
	GPAC (7)	*At. parvulum* (3)
		A. tetradius (1)
		Pn. octavius (1)
		no ID (1)
		Bacterium N14-24 (1)

GPAC, Gram-positive anaerobic cocci

obtained for 77 strains. Of these, 25 showed <98 % similarity with their closest established species (Table 6). Using these sequences a phylogenetic tree was constructed. The calculation was performed using three different treeing methods and each gave the same result for the significant branching. The phylogenetic tree obtained using the neighbour joining method is shown in Fig. 1. All sequences of *F. magna*, *P. anaerobius*, *A. tetradius*, *At. parvulum*, *Pe. niger*, *Ruminococcus gnavus* and *Bacterium* N14-24 showed ≥98 % similarity with their reference strain, while almost half of the sequences of *Pa. micra* showed <98 % similarity with their

reference strain. Also some sequences of *Pn. harei*, *A. vaginalis* and *Pn. lacrimalis* showed <98 % similarity with their closest established species. All sequences of *Pn. ivorii*, *A. lactolyticus*, *A. hydrogenalis* and *Peptostreptococcus* sp. E3_32 showed <98 % similarity with their corresponding reference strain.

Table 6. Similarity of the sequences of the clinical isolates compared with the reference strains, calculated using the DNA distance matrix. Only sequence results which yielded a near full-length sequence were used in this analysis.

	Similarity		
Strain	≥98 % (n)	<98 % (n)	Total (n)
F. magna	19	0	19
Pa. micra	9	7	16
Pn. harei	7	4	11
Pn. ivorii	0	3	3
P. anaerobius	3	0	3
A. lactolyticus	0	7	7
A. vaginalis	4	1	5
Pn. lacrimalis	1	1	2
A. tetradius	2	0	2
At. parvulum	4	0	4
Pe. niger	1	0	1
R. gnavus	1	0	1
Bacterium N14-24	1	0	1
Peptostreptococcus sp. E3_32	0	1	1
A. hydrogenalis	0	1	1

Discussion

In total 188 isolates of GPAC have been analyzed, using 16S rRNA targeted probes and/or sequencing of the 16S rRNA gene. From these results genotypic identification of the GPAC isolates was obtained. The cell morphology was used as an initial first step in the identification of GPAC, however, the results are not always unambiguous. This is illustrated by the sequence results (Table 4 and 5) that showed *At. parvulum* to be present, which is actually a coccobacilli similar to *Slackia heliotrinreducens*.

The most frequently encountered GPAC were: *F. magna* (29 %), *Pa. micra* (22 %), *Pn. harei* (18 %), *Pn. ivorii* (6 %), *A. vaginalis* (5 %), *A. lactolyticus* (5 %) and *P. anaerobius/stomatis* (5 %). *Pn. asaccharolyticus* was not observed, while it is described as being one of the most encountered GPAC in infections [32]. This is

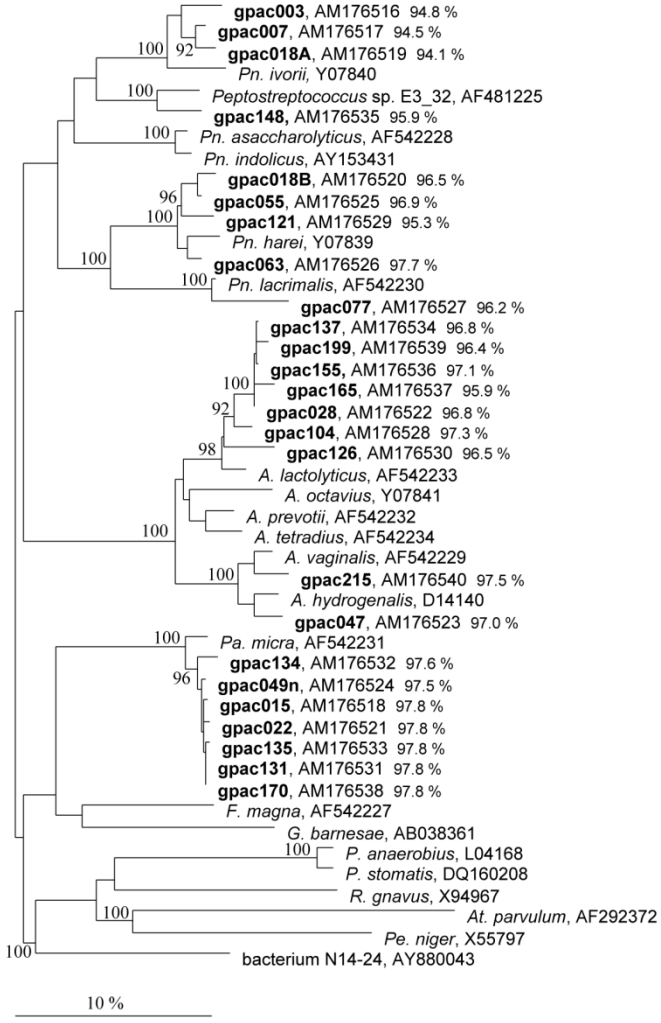

Fig. 1. Phylogenetic tree showing the relationship between GPAC and clinical isolates (shown in bold) with <98 % similarity with their closest relative. The percentages similarity were given. The neighbour joining tree was constructed using an alignment corresponding to *E. coli* basepair positions 82 to 1371. Only bootstrap values above 90 % are shown. Bar, 10 % sequence divergence.
N.B. *Peptostreptococcus* sp. E3_32 should be renamed as *Peptoniphilus* sp. E3_32.

probably due to the fact that *Pn. asaccharolyticus* can only be phenotypically distinguished from *Pn. harei* by its colony form and cell morphology [15]. Our data suggests that in the past *Pn. harei* probably has been misidentified as *Pn. asaccharolyticus*. Of all the GPAC analyzed, 18 % were genotypically identified as *Pn. harei* and is clearly a clinically relevant species which can be isolated from a variety of sites. This was also suggested by Song et al. [28]. After analyzing a selection of 190 GPAC isolates, by a multiplex PCR-assay using species-specific primers, they identified 48 of the 190 strains (25 %) as being *Pn. harei*. In our study 17 strains of *Pn. harei* were phenotypically identified, most of them as *Pn. asaccharolyticus*/ *Pn.harei*. One was misidentified as *Pn. indolicus* and three could not be identified. The four strains positively identified with the Pana134 probe were phenotypically identified as *P. anaerobius*. All strains of *F. magna* were correctly phenotypically identified. In contrast to this, *A. lactolyticus* strains were either misidentified as *F. magna* or could not be identified. Most of the *Pn. ivorii*, *Pa. micra* and *A. vaginalis* strains were also correctly identified phenotypically.

The Pana134, Pnivo731 and Avag1280 probes gave a weak hybridization signal. Fuchs et al. [10] described that by including unlabeled helper oligonucleotides during hybridization, the *in situ* accessibility of the 16S rRNA could be increased. We designed unlabeled helper oligonucleotides which bind adjacent to the probe target site (Table 2). This resulted in an increased *in situ* accessibility of the 16S rRNA and therefore in an increase of the hybridization signal. The species *F. magna*, *A. vaginalis* and *Pn. ivorii* needed permeabilization prior to hybridization. The first hybridization results with these large cocci showed a negative signal for EUB338 and the specific probes. This was resolved by treating the cells with proteinase-K to increase permeabilization prior to hybridization and recommended by us when genotypically identifying large GPAC. Cell morphology may facilitate the selection of probes for hybridization. For large cocci the Fmag1250, Avag1280 and Pnivo731 probes should be used, for middle sized cocci the probes Pnasa1254, Alac1438 and Pnhar1466, for small cocci the Pamic1435 probe and for cocci in chains the Pana134 probe. However, it is also possible to use the complete set of probes.

The Avag1280 and Alac1438 probes failed to hybridize with some of the corresponding strains, 2 out of the 10 and 1 out of the 9 strains, respectively. Analysis of the sequence data revealed mismatches of the probes with the clinical isolates. Hill et al. [12] demonstrated the heterogeneity of GPAC by analysis of 16S-23S intergenic ribosomal RNA polymorphisms. They tried to differentiate the currently recognized taxa within the group of GPAC by comparing the banding pattern obtained by amplifying the ISR of reference strains with that of test strains. They found a considerable intra-species variation for *A. vaginalis*, while all

members of *Pn. ivorii* produced identical banding patterns. No clinical isolates of *A. lactolyticus* were tested in this study. Analysis of our sequence data confirmed the intra-species variation demonstrated by Hill et al. [12]. Especially the *A. lactolyticus* group showed a large heterogeneity in their 16S rRNA sequence data. When designing species-specific probes for species with such a heterogeneity in their sequence data, the probability exists that probes will be restricted in specificity and may have mismatches with the corresponding isolates. We tried to overcome this by including the sequences of clinical isolates in the probe design and succeeded in this for most of the newly designed species-specific probes. Only the Avag1280 and Alac1438 probes showed mismatches with corresponding isolates. From the 77 strains which yielded a nearly full-length sequence 25 strains had <98 % similarity with their closest established species. This is probably due to the intra-species variation within the GPAC and they might be considered as new species. In addition, one strain isolated from abdominal fluid showed 95.9 % similarity with *Peptostreptococcus* E3_32. This strain has been found in an endodontic infection by Munson et al. [19]. A strain isolated from abdominal fluid showed 99.8 % similarity with *Bacterium* N14-24, isolated by Hooper et al. (unpublished) from oral squamous cell carcinoma tissue. These latter two strains may represent new species.

Since the newly designed probes were validated and directed against gram-positive bacteria we have chosen to perform all hybridizations overnight to obtain an optimal hybridization signal. A shorter hybridization time (2h) is also possible, however for some GPAC the hybridization signal will be less detectable compared with an overnight hybridization. With our probe set 87 % of all isolated GPAC can be identified fast and reliably. These probes make it is possible to differentiate *Pn. asaccharolyticus* from *Pn. harei*, which is difficult with phenotypic methods. Our identification method is more reliable than the phenotypic methods, especially for the species *Pn. harei*, *Pn. ivorii* and *A. lactolyticus*. Moreover, the cost of phenotypic methods is higher than for FISH. In addition the described probes have a great potential to be applied directly on clinical material for a fast and direct detection without costly anaerobic cultivation.

Acknowledgement

We would like to thank the technicians of the anaerobic workgroup and the PCR-group for their contribution in the practical work. We are indebted to Eveline Roelofsen from the Regional Public Health Laboratory in Enschede for providing clinical isolates. We thank Dr. S. Smith (Deakin University, Australia) for critical reading of the manuscript. None of the authors has commercial relationships. This

study was presented, in part, at the 8th biennial Congress of the Anaerobe Society of the Americas (Boise, 2006).

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Chapter 4

Assessment of the microbiota of a mixed infection of the tongue using phenotypic and genotypic methods simultaneously and a review of the literature

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Anaerobe 2011; 17:47-51.

Abstract

We assessed the microbiota of a tongue abscess in which twelve different aerobic and anaerobic bacteria were identified using fluorescent *in situ* hybridisation (FISH), sequencing of the 16S rRNA gene and phenotypic methods. By applying the 16S rRNA based probes directly on the clinical material, a quick insight of the bacteria present was obtained and the species which were not cultured but present in the abscess were identified.

Introduction

Tongue abscesses are rare, even though the tongue is subject to trauma. This is probably due to the extensive blood supply of the tongue, its unique muscular anatomy, the thickness of the covering mucous membrane, the cleansing action of saliva, the antimicrobial properties of saliva and the extensive lymphatic drainage of the tongue [2, 3, 14]. Tongue abscesses are more likely to occur when the immune system of the patient is impaired. Amongst others, trauma to the tongue and poor oral hygiene can predispose to the occurrence of a tongue abscess. Abscesses in the posterior part of the tongue usually originates from adjacent structures, while abscesses in the anterior part of the tongue are usually preceded by trauma [17].

We report a case of a tongue abscess and used several methods to identify most of the bacteria present in the abscess. Aerobic and anaerobic culture were used as well as molecular techniques, i.e. sequencing and fluorescent *in situ* hybridisation (FISH). We used FISH to quickly determine which of the most common oral clinically relevant bacteria were present in the tongue abscess.

Case report

A 16-year-old female patient with an extensive swelling of the tongue was referred by her family doctor to the Department of Oral and Maxillofacial Surgery of the University Medical Center Groningen. The swelling of the floor of the mouth and the tongue had increased over the last seven days (Fig 1A), despite treatment for three days with an oral antibiotic, phenethicillin, 500 mg, 3 tid, by her family doctor. The swelling was accompanied by pain and discomfort during mastication and speech. The patient reported that she had never experienced an episode of swelling of the tongue before, or to have worn a tongue piercing nor experienced other trauma to the tongue. Her oral health was good, and her dentition had no cavities. Her medical history revealed a post-streptococcal glomerulonephritis in her childhood, and chronic asthmatic bronchitis for which she was taking budesonide inhaler, cetirizine and terbutaline inhaler. She had no reported allergies, neither did she have a history of endocrine disorders.

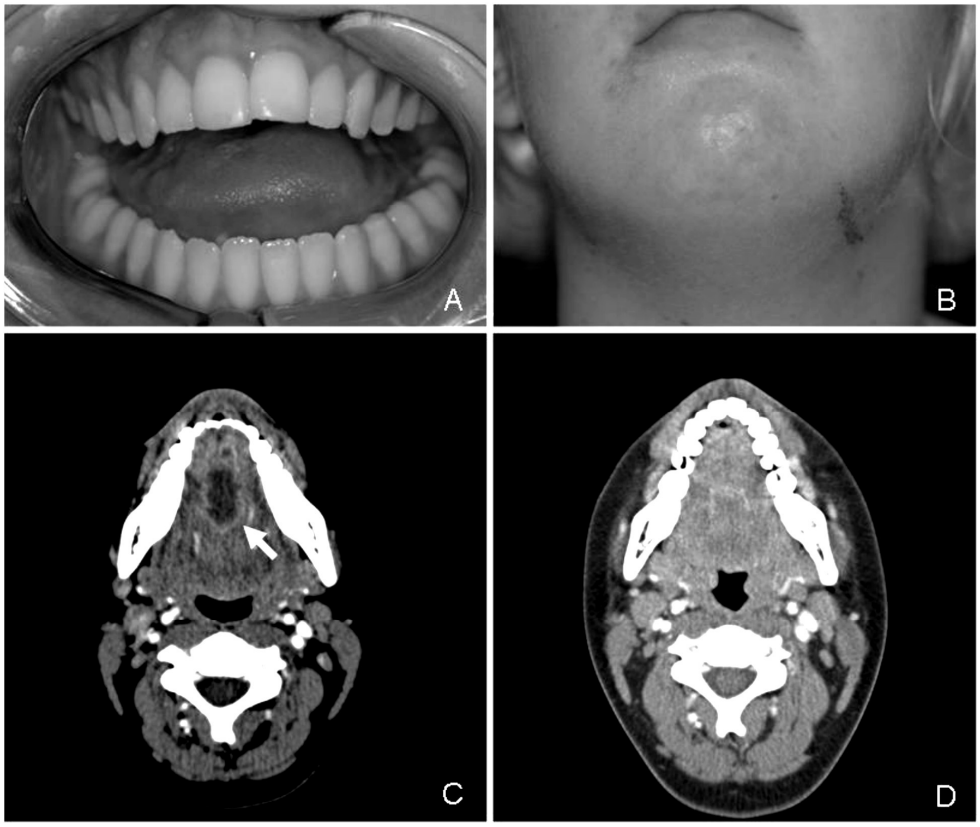

Fig. 1. A; clinical view at the time of referral. Note the elevation of the tongue and swelling of the floor of the mouth. B; clinical view at the time of referral. Note the swollen submental and submandibular region. C; contrast enhanced axial CT image made at the day of referral showing a radiolucent area central in the tongue (white arrow). D; contrast enhanced axial CT image one month after drainage of the abscess showing a normal anatomy.

Examination of the tongue and oral cavity was clearly uncomfortable to the patient. There were no signs of stridor. No ulcerations of the mucosa were present. Palpation was hardly possible due to the large size and tenderness of the tongue. The dentition showed no pathology and the submandibular glands were not swollen. No lymphadenopathy was observed, but this could have been masked by the swollen submental and submandibular regions (Fig 1B). At clinical examination, the patient's body temperature was 37.9 °C (tympanic measurement), and laboratory analysis showed an elevated white blood cell count of 12.0×10^9 /l. A computerized tomographic (CT) image of the head and neck area showed a circumscript central tongue abscess (Fig 1C).

The abscess was punctured under general anesthesia by fine-needle aspiration to examine whether a cyst or abscess could be expected on surgical exposure. Prior to collecting the aspirate for microbiological examination the oral mucosa was decontaminated using chlorhexidine mouthwash. As pus was aspirated, an incision was placed in the area of the lingual frenulum. Subsequently, the abscess space was explored by blunt dissection of the central part of the tongue. No lining of a cyst or granulomatous tissue was observed. Finally, the operated area was rinsed with NaCl and a Penrose drain was inserted which was fixed with a suture.

Post-surgery, the oral antibiotic coverage was empirically changed to intravenous administration of amoxicillin with clavulanic acid, 625 mg 3 tid. The patient recovered well and within a few days the function of the tongue was normal again. One month after surgery, a control CT showed a normal anatomy of the tongue and base of the mouth (Fig 1D).

Microbiology

The punctate of the abscess, collected in a syringe, was immediately sent to the Medical Microbiology laboratory for direct processing, which was within an hour after punctation. The gram-stain showed a variety of gram-positive cocci, gram-negative rods and some gram-positive rods. Since so many different bacteria were seen, the material was cultured aerobically and anaerobically on an extensive set of media. For the isolation of the aerobic bacteria: bloodagar (BA), chocolate agar (CHOC), McConkey agar (MC), Sabouraud dextrose agar (SAB), colistine blood agar (COB) and blood aztreonam agar (BAZ). For the isolation of anaerobic bacteria: Brucella blood agar (BBA), phenylethyl alcohol blood agar (PEA), kanamycin-vancomycin laked blood agar (KVLB) and Bacteroides bile esculin agar (BBE). For the isolation of *Actinomyces* aerobic and anaerobic incubation on a mupirocin-metronidazole blood agar (MMBA) was performed. Anaerobic culture handlings, incubation and isolation of anaerobic bacteria were performed in an anaerobic cabinet. Anaerobic plates were incubated for a week. The aerobic culture yielded *Streptococcus oralis*, *Streptococcus intermedius*, *Streptococcus constellatus* and *Haemophilus aphrophilus*. Six different anaerobic strains were recovered from the material. *Prevotella intermedia*, *Parvimonas* (*Pa.*) *micra*, *Prevotella oris*, *Actinomyces meyeri*, *Campylobacter rectus* and *Dialister pneumosintes*. Of the latter four strains and the *Streptococcus* strains, DNA was isolated as described by Boom et al. 54] and the 16S rRNA gene was amplified and sequenced using universal 16S rRNA-specific primers [11]. Obtained sequences were compared with sequences present in Genbank using the Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *H. aphrophilus* strain was identified

using RapID NH (Remel, USA). *P. intermedia* was phenotypically identified using the Wadsworth-KTL Manual [13] and *Pa. micra* using a species-specific probe [27]. Culture results are summarized in Table 2.

Table 1. Probes applied on the clinical material and/or pure culture.

Probe	Target-organism	Probe (5'- 3')	Reference
Eub338	Domain bacteria	GCTGCCTCCCGTAGGAGT	[1]
Pamic1435	*Parvimonas micra*	TGCGGTTAGATCGGCGGC	[27]
Pnhar1466	*Peptoniphilus harei*	GTCACY*TATCCTACCTTC	[27]
Alac1438	*Anaerococcus lactolyticus*	CCACAAGGGTTCGCTCAC	[27]
Pnasa1254	*Peptoniphilus asaccharolyticus*	CTATCACTAGCTCGCCCG	[27]
Fus390	*Fusobacterium* sp.	CACACAGAATTGCTGGATC	Unpublished
Bac303	*Bacteroides* sp. and *Prevotella* sp.	CCAATGTGGGGGACCTT	[16]
Bfra602	*Bacteroides fragilis* group	GAGCCGCAAACTTTCACAA	[9]
Str493	*Streptococcus* sp. and *Lactococcus* sp.	GTTAGCCGTCCCTTTCTG	[9]

* Y, an C/T nucleotide degeneracy.

Table 2. Bacteria found in the tongue abscess.

	Type of identification
Aerobic culture	
Streptococcus oralis	sequence
Streptococcus intermedius	sequence
Streptococcus constellatus	sequence
Haemophilus aphrophilus	RapID NH
Anaerobic culture	
Prevotella intermedia	phenotypically
Parvimonas micra	FISH
Prevotella oris	sequence
Actinomyces meyeri	sequence
Campylobacter rectus	sequence
Dialister pneumosintes	sequence
FISH directly on the pus	
Fusobacterium sp.	FISH
Pa. micra	FISH
Pn. harei	FISH
Prevotella sp.	FISH
Streptococcus sp.	FISH

Fluorescent *in situ* hybridisation

Pus from the abscess was directly fixed in 96 % ethanol (1:1) and stored at -20 ºC. FISH was performed as described previously [27], using a selective set of probes (Table 1). The probe selection was based on the cell morphologies observed in the gram-stain and the probes available in our laboratory. Hybridized bacteria were visualized using an epifluorescence Olympus BH2 microscope (Hamburg, Germany). Using fluorescent *in situ* hybridisation the following bacteria were detected in the clinical material: *Fusobacterium* sp., *Pa. micra*, *Peptoniphilus* (*Pn.*) *harei*, *Prevotella* sp. and *Streptococcus* sp. (Table 2, Fig 2).

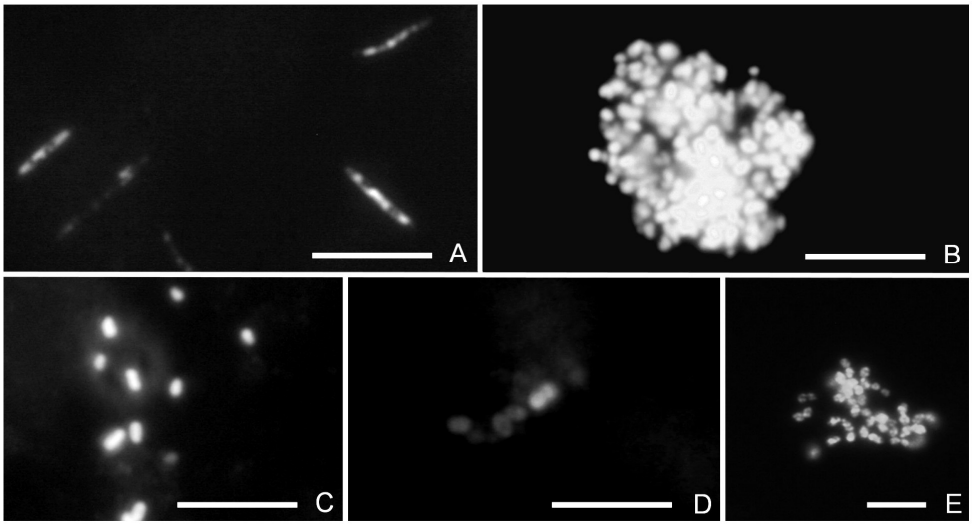

Fig. 2. Epifluorescent images of the clinical material directly hybridized with 16S rRNA based probes. A; fusobacteria hybridized with Fus390. B; *Parvimonas micra* hybridized with Pamic1435. C; *Prevotella* sp. hybridized with Bac303. D; *Peptoniphilus harei* hybridized with Pnhar1466. E; streptococci hybridized with Str493. Bar, 5 µm.

Discussion

Bernadini [4] reviewed the literature from 1816-1945 and identified 186 cases of tongue abscesses during this period. Sands et al. [22] reported one case in 1993, and identified 28 cases described in the English literature 25 years prior to their case. We identified 30 cases of tongue abscesses in the English written literature (Table 3), in which bacteriology was performed, published in the last 17 years. Published cases of tongue abscesses, e.g. caused by tongue piercing, in which no bacteriology was performed were excluded. Most of the 30 patients were male (22/30) and 7 patients had trauma to the tongue prior to abscess formation. Half of these patients (15/30) had an underlying condition which compromises the

immune system, diabetes mellitus (4/15) being the most common one. Almost half (11/30) of the patients were reported to have poor oral health.

In our case the patient had good oral health, no underlying condition and she denied any trauma to the tongue. The only predisposing factor in our case was the use of a steroid inhaler, which gives a higher risk of oral infections e.g. candidosis. Microbiological samples from the published cases yielded different results, from negative to polymicrobial with three different species of anaerobes. In most of the cases no attempts were made to identify all anaerobes present and molecular techniques were not used to identify the bacteria present. We attempted to culture all bacteria present, aerobes and anaerobes, and used FISH to rapidly identify aerobic and anaerobic pathogens. By culture we succeeded in isolating 10 different species from the abscess, 4 aerobes and 6 anaerobes (Table 2). In addition by FISH the presence of two other species was detected, *Fusobacterium* sp. and *Pn. harei*. These two species were probably overgrown by the other bacteria present, or were affected by the phenethicillin prescribed by her family doctor. In this case FISH and culture complemented each other. The detection of bacteria directly in clinical samples using FISH is limited by the fact that only bacteria can be detected for which probes are available. This explains why more bacteria were recovered by culture than with FISH.

It can be concluded that the microbiota in the abscess was complex and consisted out of at least 12 different species, mainly oral bacteria [13]. The origin of *Pn. harei* remains unclear. The commensal habitat and clinical relevance of this bacterium still has to be established. It has been described that in the past *Pn. harei* is often misidentified as *Peptoniphilus asaccharolyticus* [25]. Since *Pn. asaccharolyticus* is part of the oral flora it seems reasonable to assume that this is also the case with *Pn. harei*. However it should be noted that new species have been added to the genus *Peptoniphilus* [23]. One of these, *Pn. gorbachii* is closely related to *Pn. harei* and the *Pn. harei* probe will also react with this species. Since the abscess contained so many bacteria, and the patient received antibiotics prior to surgery, it cannot be excluded that more species were present. Furthermore, the complexity of the microbiota indicates that surgical intervention was more important than antimicrobial therapy. As on surgical exploration of the abscess no remnants of a cyst or other tissue were observed, and also the CT did not suggest presence of e.g. a dermoid cyst or median neck cyst, the origin of the abscess remains uncertain. A minor previous trauma, not noticed or not reported by the patient, cannot be excluded. To our knowledge this is the first report in which, besides culture techniques, a molecular tool was used to identify the bacteria present in a tongue abscess.

Table 2. Concise summary of tongue abscess cases found in english written literature, in which bacteriology was performed, from the last 17 years.

Case	Age	Sex	Underlying condition	Oral hygiene	Trauma to tongue	Culture	Reference
1	40	male	no	n.m.	irritation by broken molar	viridans streptococci peptostreptococci *Bacteroides ureolyticus*	[4]
2	48	male	diabetes end stage renal disease diabetic retinopathy peripheral neuropathy	n.m.	no	negative Gram-stain: few gram-positive cocci, many gram-negative rods, some filamentous organisms	[21]
3	40	male	polycythemia vera	poor	no	pigm. *Prevotella/ Porphyromonas* *Fusobacterium nucleatum* micro-aerophilic streptococci	[14]
4	51	male	no	poor	no	*S. viridans*	[14]
5	30	male	viral pharyngitis	good	no	enterococci respiratory *Neisseria*	[10]
6	47	male	alcohol abuse	poor	no	anaerobic bacteria	[18]
7	36	female	no	good	no	mixed aerobic/ anaerobic microbiota	[17]
8	23	female	subcutaneous abscesses schizophrenia mycotic emboli	n.m.	injecting heroin	*S. milleri*	[20]
9	27	male	no	poor	no	*Pasteurella multocida*	[12]
10	15	male	no	n.m.	perforation by teeth during fall	*P. melaninogenica* *F. nucleatum* *Peptostreptococcus micros*	[6]
11	55	male	alcohol abuse thyroid cancer	n.m.	during extraction of molar	*S. faecalis*	[2]

12	53	male	leukemia	poor	no	aerobic streptococci anaerobic streptococci	[2]
13	49	male	diabetes mellitus	n.m.	fishbone	*Bacteroides* sp. Gram-stain: gram-negative rods, gram-positive cocci	[2]
14	39	male	trisomy 21	poor	no	*S. agalactiae* *Klebsiella pneumoniae* subsp. *ozaenae* *Candida albicans*	[26]
15	67	female	diabetes mellitus	poor	no	*S. viridans*	[3]
16	58	male	no	poor	no	negative	[3]
17	44	male	organic psychosyndrome	poor	no	negative	[3]
18	65	male	diabetes mellitus	good	no	negative	[3]
19	44	male	lingual tonsillitis	n.m.	no	*Prevotella* sp.	[8]
20	7	male	no	n.m.	no	*Staphylococcus epidermidis*	[15]
21	14	female	no	n.m.	fishbone	negative	[15]
22	17	male	streptococcal pharyngitis	n.m.	tongue piercing	*Streptococcus* sp. group A	[7]
23-29	29-64	3 male 3 female	no	2 poor 4 good	no	1 anaerobic bacteria 1 *Streptococcus viridans* 4 negative	[19]
30	22	male	pharyngitis	n.m.	no	Gram-positive cocci anaerobes	[24]

* Not mentioned.

Nomenclature of bacteria is from the original publication.

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Chapter 5

The mistaken identity of

Peptoniphilus asaccharolyticus

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J. Clin. Microbiol. 2011; 49:1189.

Data letter

Peptoniphilus (*Pn.*) *asaccharolyticus* is a commonly isolated gram-positive anaerobic coccus (GPAC) [7]. However, the type strain ATCC14963 is not representative for the species. Huss et al. [4] described that the DNA-DNA homology between the type strain and clinical isolates was < 25%.

Because of this finding new species were described [6], among them *Pn. harei*. *Pn. harei* has the same biochemical features as *Pn. asaccharolyticus* and can only be differentiated from *Pn. asaccharolyticus* by its irregular colony and cell morphology [5]. The clinical relevance of *P. harei* was unknown. However, in studies of clinical isolates using molecular techniques for identification a remarkable number of *Pn. harei* was found. Song et al. [9] identified 25.3 % of all GPAC as *Pn. harei*. In another study [10] 17.0 % was identified as *Pn. harei* by fluorescent *in situ* hybridisation. In both studies, no *Pn. asaccharolyticus* was encountered. To substantiate the genotypic identity of *Pn. asaccharolyticus* reference strains were needed. To this end, a number of type strains were re-identified using 16S rRNA gene sequencing. These were *Pn. asaccharolyticus* strains from the Culture Collection of the University of Göteburg (Sweden) CCUG42643, CCUG43862, CCUG44165, CCUG47015, and CCUG48151. DNA was isolated and amplified as described [1, 2]. Sequences were aligned and a filter was set at *Escherichia coli* positions 257 and 1436. Sequence similarities with closest relatives and *Pn. asaccharolyticus* were calculated using the DNA distance matrix in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

For each strain the closest relative was *Pn. harei*, with sequence similarities between 99.0 and 99.4 % (Table 1). The sequence similarity with *Pn. asaccharolyticus* was between 89.2 and 89.6 %. The original identification of these strains was based on their biochemical features. Since, *Pn. harei* and *Pn. asaccharolyticus* share the same biochemical features it is clear that these strains were misidentified in the past.

Song et al. [8] developed a flow chart for the phenotypical identification of GPAC. It is mentioned that the alkaline phosphatase test might be useful to differentiate the species from each other. The sequence similarity of *Pn. asaccharolyticus* strain ATCC29743 with *Pn. harei* was 99.6 % (Table 1), indicating that it is not *Pn. asaccharolyticus*. However, in the study of Song et al. [8] this strain was assumed to be *Pn. asaccharolyticus*. This confirms that *Pn. harei* and *Pn. asaccharolyticus* cannot be differentiated from each other phenotypically. Holdeman-Moore et al. [3] commented already in 1986 that one should be cautious in reporting on isolation and incidence of *Pn. asaccharolyticus*. In our opinion this caution still stands. The fact that the type strain of *Pn. asaccharolyticus* ATCC14963 is atypical for clinical isolates might be due to the true identity of the

clinical isolates used for comparison. This can explain the low DNA-DNA homology [4] between the type strain and clinical isolates.

We are convinced that the incidence of *Pn. asaccharolyticus* in clinical material is highly overestimated. The clinical importance of *Pn. harei* in the pathogenesis of anaerobic infections still has to be defined.

The 16S rRNA sequences of strains of CCUG42643, CCUG43862, CCUG44165, CCUG47015, and CCUG48151 have been deposited in Genbank under accession numbers HQ326629, HQ326630, HQ326631, HQ326632, and HQ326633, respectively.

Table 1. Sequence similarities of the 16S rRNA genes between the type strains of *Pn. harei* and *Pn. asaccharolyticus,* and several strains which were originally identified as *Pn. asaccharolyticus*.

Strain	% similarity	
	Pn. harei ATCC BAA-601^T	*Pn. asaccharolyticus* ATCC 14963^T
ATCC 29743 (DQ986463)	99.4	89.5
CCUG 42643 (HQ326629)	99.1	89.2
CCUG 43862 (HQ326630)	99.0	89.4
CCUG 44165 (HQ326631)	99.3	89.4
CCUG 47015 (HQ326632)	99.2	89.3
CCUG 48151 (HQ326633)	99.3	89.3
Pn. harei ATCC BAA-601^T	100	89.6

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Chapter 6

Identification of gram-positive anaerobic cocci by MALDI-TOF Mass Spectrometry

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Syst. Appl. Microbiol. 2011; 34:58-62.

Abstract

Gram-positive anaerobic cocci (GPAC) are part of the commensal microbiota of humans and are a phylogenetically heterogeneous group of organisms. To evaluate the suitability of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) for the identification of GPAC, a database was constructed, using reference strains of commonly encountered GPAC and clinical isolates of which the sequence of the 16S rRNA gene was determined. Subsequently, the database was validated by identifying 107 clinical isolates of GPAC. Results were compared with the identifications obtained by 16S sequencing or fluorescent *in situ* hybridization (FISH). Strains belonging to the same species grouped together, in most cases, by MALDI-TOF MS analyses. Strains with sequence similarities less than 98 % to their closest relatives, formed clusters distinct from recognized species in the MALDI-TOF MS dendrogram and, therefore could not be identified. These strains probably represent new species. Only three clinical isolates (2 strains of *Finegoldia magna* and 1 strain of *Anaerococcus vaginalis*) could not be identified. For all the other GPAC strains (96/107), reliable identifications were obtained. Therefore, we concluded that MALDI-TOF MS is an excellent tool for the identification of phylogenetically heterogeneous groups of micro-organisms such as GPAC.

Introduction

Gram-positive anaerobic cocci (GPAC) are part of the commensal human microbiota and are known to play an important role in human disease. They account for about one third of all anaerobes recovered from human clinical specimens [13]. It is a heterogeneous group of organisms and, therefore, the taxonomy has changed extensively. The species *Peptostreptococcus micros* and *Peptostreptococcus magnus* have been transferred to different genera; *Micromonas* and *Finegoldia*, respectively [14], with *Micromonas micros* and *Finegoldia magna* the only species currently present in each genus. The genus *Micromonas* was replaced by *Parvimonas*, since the name *Micromonas* was ruled to be illegimate [21]. *Parvimonas* (*Pa.*) *micra* is the only species present in this genus. The remaining peptostreptococci have been divided into three phylogenetic groups [4], *Peptoniphilus* gen. nov., *Anaerococcus* gen. nov. and *Gallicola* gen. nov., with *Gallicola barnesae* the only species present in the latter genus. The only species not reclassified in the genus *Peptostreptococcus* was *P. anaerobius*, although more recently *P. stomatis*, isolated from the human oral cavity, was added [3]. Murdoch et al. [12], described a number of species that were first assigned to the genus *Peptostreptococcus*, but were later reclassified as *Peptoniphilus* (*Pn.*) *harei*, *Pn. ivorii* and *Anaerococcus octavius*. Recently, Song et al. [17] described

three new species; *Anaerococcus murdochii*, *Pn. gorbachii* and *Pn. olsenii*. The addition of all these new species illustrates the heterogeneity of GPAC.

The phenotypic identifications of GPAC for certain species are not always reliable [22], for example, *Pn. asaccharolyticus* cannot be phenotypically differentiated from *Pn. harei* [7] since they share the same biochemical features. In the past, *Pn. harei* has often been misidentified as *Pn. asaccharolyticus* [22], which resulted in an over-estimation of the clinical relevance of *Pn. asaccharolyticus*. To overcome these problems, molecular methods have been applied to aid the reliable identification of GPAC. Song et al. [18] developed a multiplex PCR assay for the identification of GPAC, using genus- and species-specific primers. Wildeboer-Veloo et al. [22] developed species-specific 16S rRNA-based probes for the identification of GPAC. Both methods yield reliable identifications. Recently, another technique is increasingly used for the identification of bacteria: Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Several studies have been performed in which MALDI-TOF MS has been used for the identification of anaerobic bacteria [5, 15, 20]. In all these studies, MALDI-TOF MS was assessed to be superior to conventional phenotypic identification, for example *Prevotella intermedia* can be differentiated from *Prevotella nigrescens* [20], even though both share the same biochemical features. However, the reliability of identification, using MALDI-TOF MS, depends on whether the mass spectra of the species are present in the database [5, 9, 16]. In order to validate the suitability of MALDI-TOF MS for the identification of GPAC, a database was established, using reference strains and clinical isolates of which the sequence of the 16S rRNA gene was determined. Subsequently, unknown clinical isolates of GPAC were identified independently by mass spectral and molecular approaches.

Material and methods

Bacterial strains

All strains, except the reference strains, were isolated from human clinical samples obtained from a variety of anatomical sites,i.e., from abdominal, head and neck and soft tissue infections. Strains, stored at – 80 ºC until use, were cultured on Brucella Blood Agar (BBA) at 35 ºC, in an anaerobic chamber. Subsequently, they were sent to AnagnosTec (Golm, Germany) on a swab in a suitable transport medium (Transwab®, Medical Wire & equipment, England). On arrival, strains were immediately subcultured on BBA.

Table 1. Reference strains and clinical isolates of GPAC used to construct a database for MALDI-TOF MS identification.

Strains	no. of strains
Reference strains	
Pa. micra DSM204468^T	
A. vaginalis DSM7457^T	
F. magna DSM20470^T	
Pn. harei DSM10020^T	
Pn. ivorii DSM10022^T	
Pn. gorbachii DSM21461^T	
A. lactolyticus DSM7456^T	
A. murdochii DSM21462^T	
P. anaerobius DSM2949^T	
Pn. asaccharolyticus DSM20463^T	
Pn. indolicus DSM20464^T	
Pn. olsenii DSM21460^T	
Clinical isolates	
F. magna	23
Pa. micra	13
Pn. harei	12
A. vaginalis	6
Pn. ivorii[a]	5
P. anaerobius	4
Pn. gorbachii	3
Pn. lacrimalis	3
Atopobium (*At.*) *parvulum*	2
A. tetradius	1
Ruminococcus gnavus	1
Peptococcus (*Pe.*) *niger*	1
GPAC[b]	3

[a] All 5 clinical isolates had sequence similarities of less than 95 % with their closest relative, *Pn. ivorii*.

[b] No species name could be assigned since the sequence similarities of the strains showed that they were phylogenetically ambiguous.

Identification

The database for identification of GPAC, using MALDI-TOF MS, was constructed using 12 reference strains and 77 sequenced clinical isolates (Table 1). DNA of the strains was isolated as described previously [2] and the 16S rRNA genes were amplified and sequenced using universal 16S rRNA-specific primers [6]. The constructed database was validated by identifying 107 unknown clinical isolates. Identification was confirmed by fluorescent *in situ* hybridisation (FISH),

using species-specific 16S rRNA-based probes [22], directed against *P. anaerobius/stomatis*, *Pa. micra*, *F. magna*, *Pn. asaccharolyticus*, *Pn. ivorii*, *Pn. harei*, *A. vaginalis* and *A. lactolyticus*. The addition of new species to the genera *Peptoniphilus* and *Anaerococcus* [17], showed that the probes directed against *A. lactolyticus* and *Pn. harei* were also positive with *A. murdochii* and *Pn. gorbachii*, respectively (data not shown). Strains which were negative with the probes or positive with the probes directed against *A. lactolyticus* and *Pn. harei* were identified by 16S rRNA gene sequence analyses.

Sequence analysis

The 16S rRNA gene sequences of clinical isolates were determined and aligned against the sequences of reference strains obtained from the EMBL database and the Ribosomal Database Project (RDP) [11], using the ARB-software [10]. Isolates were matched to the closest related species. Similarities of the sequences of the clinical isolates with those of their closest established species were calculated, using a DNA distance matrix embedded in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide positions 167 and 1341 *Escherichia coli* 16S rRNA gene sequence numbering were included, to ensure accurate determination of species similarity. A phylogenetic tree was constructed, using the same alignment, by the neighbour-joining method with Jukes and Cantor correction, based on a distance matrix that included only positions with more than 50 % conservation and parsimony, implemented in the ARB software. The topology of the tree was calculated, following bootstrap analyses of 1,000 replicate trees.

Sample preparation

Cells from individual colonies were transferred from agar media onto a stainless steel template. To each sample, 0.5 μl matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in acetonitrile: ethanol: water (1:1:1) with 0.3 % trifluoro acetic acid) was added. Evaporation of the solvent and crystallization was performed at room temperature. All strains were analyzed in duplicate.

Measurement and data analysis

The MALDI-TOF MS measurements were performed on an AXIMA Confidence (Shimadzu). Mass spectra were acquired in a linear positive ion extraction mode, using an acceleration of 20 kV and a low mass gate of 1,500 Da. The system was calibrated externally with the mass spectrum obtained from fresh cells of an *E. coli* K12 strain CCUG 10979 [8]. Spectra were accumulated from 1,000 laser pulse cycles, automatically processed with the Shimadzu Biotech Launchpad software,

exported to and analyzed with the SARAMIS software package (AnagnosTec, Golm, Germany). Cluster analysis was performed by computing a dendrogram based on similarities of mass spectra patterns (3-20 kDa) using single-linkage agglomerative clustering and allowing for an analytical error of 0.08%. Species were separated by setting a similarity threshold at 50 % matching peaks.

Results

Database construction

For all strains, mass spectra of good quality were obtained, using the rapid preparation method mentioned above, i.e., with a resolution more than 600 and 70-150 automatically detected peaks. The number of peaks was sufficient to yield a taxon specific peak pattern, which could be used for species identification by computing species specific identifying spectra, so called 'SuperSpectra', with SARAMIS (Anagnostec, Germany). A cluster analysis performed on the mass spectra showed that isolates of the same species generally clustered together (Fig. 1B). However, some isolates had less than 98 % 16S rRNA gene sequence similarities with their closest relatives or exhibited ambiguous phylogenetic positions in between two species (Table 1, Fig. 1A), indicating that the taxonomy of GPAC is not completely settled. The isolates most closely related to *Pn. ivorii* exhibited sequence similarities less than 95 % with the type strain of the species, DSM 10022. Consequently, these isolates were not grouped with *Pn. ivorii* DSM 10022 in the MALDI-TOF MS dendrogram (Fig.1B). Likewise, isolates that could not be assigned to a described species based on 16S rRNA sequences were grouped separately from species-specific clusters (gpac104, gpac047, and gpac121). In general, all sequenced clinical isolates with sequence similarities more than 98 % grouped in a single cluster, together with corresponding reference strains (*Pa. micra*, *A. vaginalis*, *F. magna*, *P. anaerobius*). An exception was found for the clinical isolates of *Pn. harei*, that were grouped in a homogeneous cluster, at the base of which the type strain (DSM 10020) is placed with a low spectral similarity of less than 50% matching peaks. Also, one clinical isolate of *F. magna* (gpac129) did not group with the *F. magna* type strain (Fig. 1B).

Validation database

A total of 107 unknown clinical isolates of GPAC was used to test the performance of a mass spectral approach for the identification of GPAC. The results are summarized in Table 2. According to their genotypic identification, all clinical isolates of *Pa. micra*, *Pn. harei*, *A. murdochii*, *P. anaerobius*, *Pn. gorbachii*, *At. parvulum*, *Pe. niger*, *A. lactolyticus*, *A. tetradius* and *Pn. lacrimalis* were

Table 2. Clinical isolates used to validate the constructed database.

Strains (n) genotypically identified	MALDI-TOF ID (n) correct ID	no ID
F. magna (32)	29	3
Pa. micra (29)	29	
Pn. harei (15)	15	
A. murdochii (6)	6	
P. anaerobius (4)	4	
A. vaginalis (3)	2	1
Pn. ivorii (3)	1	2
Pn. gorbachii (3)	3	
At. parvulum (3)	3	
Pe. niger (1)	1	
A. lactolyticus (1)	1	
A. tetradius (1)	1	
Pn. lacrimalis (1)	1	
Pn. octavius (1)[a]		1
Bacterium N14-24 (1)[a]		1
GPAC (3)[a,b]		3

[a] Strains could not be identified by MALDI-TOF MS because no reference strains were included in the database. In the MALDI-TOF MS dendrogram, respective strains are not grouped in species-specific clusters.

[b] No species name could be assigned since the sequence similarities of the strains showed that they were phylogenetically ambiguous.

correctly identified. Of the 32 *F. magna* strains, 3 strains could not be identified. One of these strains was identified only by FISH, the other two had 16S rRNA gene sequence similarities of 97.8 % and 99.7 % with the reference strains of *F. magna*. However, when comparing mass spectra fingerprints, all three strains had less than 50% spectral similarity to all other *F. magna* strains, including the type strain.

Of the 3 *A. vaginalis* strains analyzed, one could not be identified (gpac203). The 16S rRNA gene sequence similarity of this strain with that of the reference strain of *A. vaginalis* was 98.2 %. One strain of *Pn. ivorii* was correctly identified by MALDI-TOF MS, while two other clinical isolates of *Pn. ivorii* could not be identified. However, the strain that was identified by MALDI-TOF MS had a sequence similarity with the reference strain of *Pn. ivorii* of 99.9 %, while the other 2 strains showed similarities of less than 95 %. The strains that formed a well-supported cluster in the phylogenetic tree, including the type strain DSM 10022 (Fig. 1A), were grouped in two loose clusters in the MALDI dendrogram unrelated to the type strain

No species name could be assigned to three clinical isolates (gpac126, gpac202, and gpac 215), using 16S rRNA gene sequences and these also could not be identified using the SARAMIS MALDI-TOF MS database. Two other clinical isolates, bacterium N14-24 and *Pn. octavius*, were identified as species that were not present in the database at the time of the study. As expected, these isolates could not be identified.

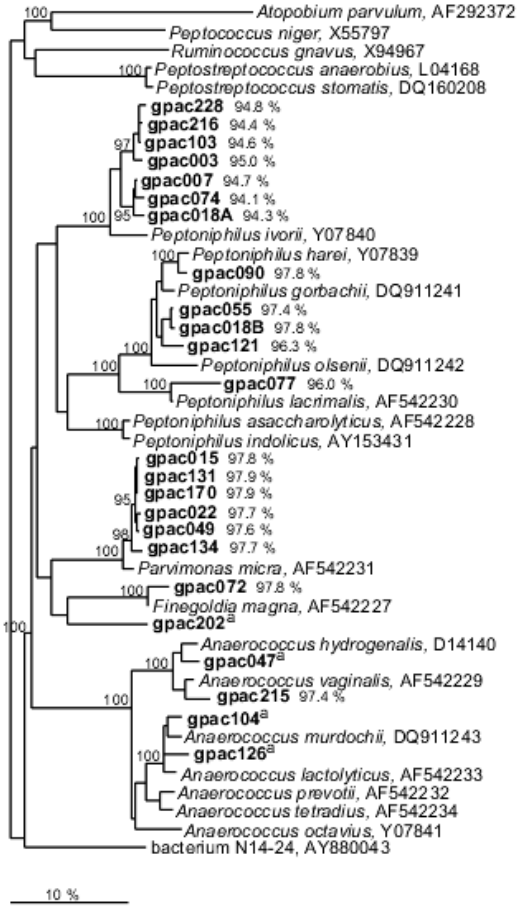

Fig. 1A. Phylogenetic tree based on 16S rDNA gene sequences showing the relationships between gram-positive anaerobic cocci, including clinical isolates (shown in bold), with less than 98% similarity to their closest relatives. The percentages of similarity are indicated. The neighbour-joining tree was constructed, using an alignment corresponding to *Escherichia coli* base-pair positions 167–1341. Only bootstrap values more than 90% are shown. The bar indicates 10% sequence divergence.

[a] Clinical isolates with no percentage of similarity were identified as GPAC, since they were phylogenetically ambiguous.

Fig. 1B. Dendrogram of all the strains used for database construction, complemented with strains from the validation which had a less than 98% sequence similarity with their closest relative. For all strains, mass spectra fingerprints of duplicate analyses were used to compute the dendrogram, using a single linkage agglomerative clustering algorithm. The numbered clusters contained exclusively the species indicated. For clarity, strain numbers are given only for strains that appear in Fig. 1A, for type strains or strains that are discussed in the text.

Discussion

Several studies have been performed in which MALDI-TOF MS has been used to identify anaerobic bacteria; *Bacteroides* sp. [15], clostridia [5] and oral anaerobic bacteria from subgingival biofilm [20]. In this study, we evaluated the possibility of MALDI-TOF MS to identify GPAC, a phylogenetically heterogeneous group of organisms.

The best performance for species identifications was for species that appear homogeneous by 16S rRNA sequence analyses as well as by mass spectral patterns; *Pa. micra*, *Pn. harei*, *A. murdochii*, *P. anaerobius*, *Pn. gorbachii*, *A. parvulum*, *Pe. niger*, *A. lactolyticus*, *A. tetradius* and *Pn. lacrimalis*. Particularly, *Pa. micra* and *Pn. harei*, the two species for which a larger number of strains could be studied, can be considered to be relatively homogeneous species. In contrast, in the cases of all species for which representative isolates were not correctly identified by MALDI-TOF MS, a higher intra-species variation, in terms of mass spectral patterns, is obvious. However one uncertainty could arise from the fact, that, as in the case of *Pn. harei*, the type strain is considered to be not typical for the species. The type strain did not appear in the same cluster as the clinical isolates.

In general, clinical isolates with sequence similarities less than 98 % with a corresponding reference strain did not appear in a cluster with a reference strain. Only one strain of *Pn. ivorii* with a high similarity of 99.9 % appeared in the same cluster as the reference strain. Among seven strains that were not identified as *Pn. ivorii*, two strains formed their own cluster (gpac007 and gpac074), having a sequence similarity with each other of 99.3 %. Three other strains (gpac003, gpac103 and gpac 216) also formed their own cluster, with sequence similarities between 96.3 % and 98.5 %. Two strains (gpac018A and gpac228) were singular. Similar relationships between the strains were established from mass spectral patterns: a first cluster consisted of strains gpac003, gpac103, and gpac216, a second of strains gpac074 and gpac007 clustering together, and two strains (gpac018A and gpac228) being singular (Fig. 1A and 1B). Low 16S rRNA gene sequence and MALDI-TOF MS similarities, compared to those of the type strain of *Pn. ivorii* suggest that the strains likely represent one or more separate species.

The similar topology of trees computed based on 16S ribosomal RNA sequences and MALDI-TOF MS patterns, can be explained by the fact that, among other proteins, many of the peaks in the spectra are due to ribosomal proteins [1]. Stackebrandt et al. [19] showed that spectral profiles, consisting of about 25 to 45 mass signals ranging between 2 and 20 kDa, do indeed have taxonomic significance. Since bacterial genera evolve at different speeds, it is difficult to set a general cutoff value for the 16S rRNA gene sequence similarity that will be

applicable for all new species. Therefore, the cutoff value may differ for the species of different genera. Since the intra-species variation for the GPAC is large, we have chosen to use a sequence similarity of more than 98 % as one species and less than 98 % sequence similarity as new species.

A similar difficulty to establish a clear cutoff value separating individual species is evident for mass spectral analyse. From the dendrogram (Fig. 1B), it is evident that intra-specific variation could result in spectral similarities as low as 50%. Therefore, this value could be set as cutoff for the separation of species. If this was done and strains that have a spectral similarity below 50% to any established species would consequently be considered to belong to other, possibly new species. A number of isolates of the present study fall into this category.

Possible new species or sub-species that were recognized based on 16S rRNA sequences (Fig. 1A) were also confirmed in the MALDI-TOF MS dendrogram (Fig. 1B). Besides the strains related to *Pn. ivorii* (mentioned above), two clinical isolates of *Pn. gorbachii* (gpac055 and gpac018B) had sequence similarities less than 98 % with the reference strain of *Pn. gorbachii*. These two strains are in the same cluster in the MALDI-TOF MS dendrogram and neighboring the cluster containing the reference strain (Fig. 1B). The same clustering can be seen in the phylogenetic tree (Fig. 1A). These strains might represent new (sub)species.

In summary, using the constructed MALDI-TOF MS database, 11 of the 107 strains could not be identified, most of them differing also in the respective 16S rRNA sequences from the closest reference strain to a degree suggesting that they actually belong to other species. Once a database has been established that comprehensively covers the intra-species variation, mass spectral identification should be accurate and reproducible. Therefore, we can conclude that MALDI-TOF MS will be an excellent tool for the identification of GPAC in clinical routine diagnostics.

The performance of the respective commercial systems, like SARAMIS or Biotyper, however, depends largely on a consistent and exhaustive taxonomy based on phylogenetic relationships, at least, for the particular field of application, like clinical microbiology. The present study produced evidence that among clinically relevant GPAC a number of new species should be considered. When respective isolates would be encountered, an identification would not be possible and alternative methods, like 16S gene sequencing, need to be applied.

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Chapter 7

Antimicrobial susceptibility of clinically relevant gram-positive anaerobic cocci, collected in a 3-year period in the Netherlands

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Antimicrob. Agents Chemother. 2011; 55:1199-1203.

Abstract

The susceptibility of 14 species of 115 gram-positive anaerobic cocci (GPAC) was determined for 14 antibiotics. To assure correct identification, strains were genotypically identified by fluorescent *in situ* hybridisation and sequencing. Susceptibility differences (MIC_{50} and MIC_{90}) for penicillin G, clindamycin, tigecycline, levofloxacin, amoxicillin-clavulanic acid, cefoxitin, ertapenem, meropenem, metronidazole, and doxycycline were found for the three clinically most relevant GPAC species; *Finegoldia magna*, *Parvimonas micra*, and *Peptoniphilus harei*.

Introduction

Gram-positive anaerobic cocci (GPAC) are part of the commensal microbiota and account for about one-third of the anaerobic isolates recovered from clinical materials [14]. It is a heterogeneous group, which in the last decade has undergone an extensive taxonomic change. The species *Peptostreptococcus micros* and *Peptostreptococcus magnus* were transferred to two new genera, *Micromonas* and *Finegoldia*, respectively, with each being the only species present in their respective genus [15]. The genus *Micromonas* has recently been replaced by *Parvimonas*, with *Parvimonas* (*Pa.*) *micra* the only species present [19]. Ezaki et al. [7] divided the remaining peptostreptococci in three phylogenetic groups, *Peptoniphilus* gen. nov., *Anaerococcus* gen. nov. and *Gallicola* gen. nov, with *Gallicola barnesae* being the only species present in the latter genus. The species left in the genus *Peptostreptococcus* is *P. anaerobius*, and a recently described new species *P. stomatis* [6]. Song et al. [18] described three new species, *Peptoniphilus* (*Pn.*) *gorbachii* sp. nov., *Pn. olsenii* sp. nov. and *Anaerococcus murdochii* sp. nov. The most commonly found GPAC in clinical material are *F. magna*, *Pa. micra*, *Pn. harei* [20] and *P. anaerobius* [21]. Data on antimicrobial susceptibility of the different species of GPAC is often based on GPAC in general, eventhough several authors describe a difference in antimicrobial susceptibility between species [3-5, 11, 12, 17]. In these studies, the strains were identified phenotypically. However, for some species it is difficult to obtain a reliable phenotypic identification e.g. in the past *Pn. harei* has been often misidentified as *Pn. asaccharolyticus* [20] probably caused by the fact that these two species share the same biochemical characteristics [10].

In this study, we have assessed the susceptibility of 115 isolates of GPAC, against 14 different antibiotics. Isolates were genotypically identified using fluorescent *in situ* hybridisation (FISH) [20] or sequencing, thus obtaining a more accurate insight in the distribution of susceptible and resistant strains within the different species.

Material and methods

Isolates

Strains were obtained from the diagnostic laboratory of the University Medical Center Groningen and collected in the years 2002-2004. All strains were isolated from human clinical samples from a variety of anatomical sites, e.g. from abdominal, head and neck and soft tissue infections. Strains were stored at -80 ºC and subcultured on Brucella Blood Agar (BBA) prior to susceptibility testing.

Identification

Strains were genotypically identified using 16S rRNA based probes [20] and sequencing. Shortly, bacterial cells were harvested from BBA using a sterile loop and fixed in 1:1 phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter) and ethanol 96 % v/v. Fixed cells were spotted on slides, and if necessary permeabilized using proteinase-K. Strains were hybridized using probes directed against *F. magna*, *Pa. micra*, *Pn. harei*, *P. anaerobius, A. vaginalis*, *Pn. asaccharolyticus*, *A. lactolyticus* and *Pn. ivorii*. The addition of new species to the genera *Peptoniphilus* and *Anaerococcus* [18], showed that the probes directed against *A. lactolyticus* and *Pn. harei* were also positive with *A. murdochii* and *Pn. gorbachii*, respectively (data not shown). Strains which were negative with the probes or positive with the probes directed against *A. lactolyticus* and *Pn. harei* were sequenced. DNA was isolated as described previously [2] and the 16S genes were amplified and sequenced using universal 16S rRNA- specific primers [9]. Sequences were compared to those in the GenBank database by performing a BLAST-search from the National Center of Biotechnology Information [1].

Susceptibility testing

The antimicrobial susceptibility against penicillin G, amoxicilin-clavulanic acid, cefotetan, cefoxitin, ertapenem, meropenem, levofloxacin, moxifloxacin, clindamycin, metronidazole, linezolid, chloramphenicol, doxycycline and tigecycline was determined using E-test (AB Biodisk, Sweden). Suspensions of approximately 2 McFarland were made in pre-reduced Brucella broth and applied onto a pre-reduced BBA. All culture handlings were performed in an anaerobic chamber. Plates with E-test strips were incubated for 48 hrs at 37 ºC, in an anaerobic chamber before reading the minimal inhibitory concentration (MIC). In each batch a quality control strain *Bacteroides fragilis* ATCC 25285 was included.

A difference in susceptibility was defined as at least 2 dilution steps (with one dilution step being a difference of two-fold dilutions with a precision of a 0.5 dilution) difference between the MIC's of the different species.

Results

The quality control strain *B. fragilis* ATCC 25285 was tested 10 times with all 14 antibiotics. The obtained MIC's are summarized in Table 1.

Table 1. MIC-values of the quality control tests on *Bacteroides fragilis* ATCC 25285

	MIC-value (no. of tests)	Expected range[1]
Penicillin G	12 (2), 16 (7), 24 (1)	8-32
Amoxicillin/clavulanic acid	0.19 (2), 0.25 (5), 0.38 (3)	0.125-0.5[†]
Cefotetan	6 (7), 8 (3)	4-16
Cefoxitin	4 (1), 6 (7), 8 (2)	4-16
Ertapenem	0.125 (4), 0.19 (6)	0.064-0.25
Meropenem	0.094 (2), 0.125 (4), 0.19 (4)	0.064-0.25
Levofloxacin	1 (1), 1.5 (9)	1[*]
Moxifloxacin	0.19 (1), 0.25 (1), 0.38 (6), 0.5 (2)	0.125-0.5
Clindamycin	1.5 (2), 2 (4), 3 (4)	0.5-2
Metronidazole	0.25 (4), 0.38 (4), 0.5 (2)	0.25-1
Linezolid	4 (1), 6 (5), 8 (3), 12 (1)	2-8[*]
Chloramphenicol	6 (3), 8 (7)	2-8
Doxycycline	0.25 (3), 0.38 (5), 0.5 (2)	0.25-0.5[*]
Tigecycline	0.25 (2), 0.5 (2), 0.75 (6)	0.125-1[*]

[1] The expected range is derived from CLSI for *B. fragilis* for reference agar dilution testing, unless indicated with a * or †.

† Expected range derived from the manufacturer.

* Expected range derived from literature.

All results of the clinical isolates are summarized in Table 2 and Table 3. The MIC_{50} and MIC_{90} were only calculated for species of which more than 10 strains were present in the study, i.e. *F. magna*, *Pa. micra* and *Pn. harei*. Comparing the MIC_{50} and MIC_{90} of these 3 species with each other, *F. magna* shows the highest MIC_{50} and MIC_{90} values for penicillin G, amoxicillin-clavulanic acid, clindamycin,and tigecycline. It has the highest MIC_{50} values for cefotetan, cefoxitin, meropenem, linezolid and chloramphenicol and the highest MIC_{90} values for levofloxacin and moxifloxacin. *Pa. micra* has the lowest MIC_{50} and MIC_{90} for levofloxacin, metronidazole and doxycycline. It has the lowest MIC_{90} for amoxicillin-clavulanic acid. *Pn. harei* has the highest MIC_{50} for levofloxacin and doxycycline. It has the lowest MIC_{50} and MIC_{90} for cefoxitin, ertapenem and meropenem and the lowest MIC_{90} for chloramphenicol.

Table 2. MIC's and range for GPAC against 14 antibiotics.

Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}	Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}
F. magna (31)	Penicillin G	0.023 - 0.38	0.125	0.25	*Pn. harei* (16)	Penicillin G	0.016 – 0.19	0.023	0.032
	Amoxicillin-clavulanic acid	0.094 – 2	0.25	0.5		Amoxicillin/clavulanic acid	0.016 – 0.38	0.023	0.25
	Cefotetan	0.25 – 4	2	2		Cefotetan	0.38 - 8	0.5	1
	Cefoxitin	0.38 – 3	1	1.5		Cefoxitin	0.023 – 1.5	0.094	0.5
	Ertapenem	0.016 – 0.19	0.064	0.125		Ertapenem	0.006 – 0.023	0.012	0.016
	Meropenem	0.064 – 0.25	0.125	0.19		Meropenem	0.004 – 0.032	0.008	0.032
	Levofloxacin	0.094 – 64	0.75	64		Levofloxacin	2 – 64	4	6
	Moxifloxacin	0.047 – 64	0.19	6		Moxifloxacin	0.125 – 1.5	0.19	0.38
	Clindamycin	0.125 - >256	1	3		Clindamycin	0.094 – 1.5	0.25	1.5
	Metronidazole	0.094 – 1.5	0.38	1		Metronidazole	0.032 – 2	0.38	1.5
	Linezolid	2 – 6	3	3		Linezolid	0.5 – 2	0.75	1.5
	Chloramphenicol	4 – 16	6	8		Chloramphenicol	1.5 – 4	3	3
	Doxycycline	0.75 – 24	2	24		Doxycycline	0.064 – 24	8	16
	Tigecycline	0.064 – 1	0.25	0.75		Tigecycline	0.023 – 0.25	0.094	0.25
Pa. micra (27)	Penicillin G	0.016 – 0.125	0.016	0.047	*A. vaginalis* (8)	Penicillin G	0.016 – 0.094		
	Amoxicillin/clavulanic acid	0.016 – 0.75	0.032	0.094		Amoxicillin/clavulanic acid	0.016 – 0.125		
	Cefotetan	0.125 - 2	0.38	1.5		Cefotetan	0.094 – 0.5		
	Cefoxitin	0.125 - 3	0.5	2		Cefoxitin	0.032 – 0.125		
	Ertapenem	0.008 – 0.19	0.047	0.125		Ertapenem	0.023 – 0.19		
	Meropenem	0.008 – 0.38	0.047	0.19		Meropenem	0.006 – 0.125		
	Levofloxacin	0.125 – 3	0.25	0.5		Levofloxacin	24 – 64		
	Moxifloxacin	0.094 – 1.5	0.19	0.38		Moxifloxacin	0.5 – 2		
	Clindamycin	0.047 – 2	0.38	1.5		Clindamycin	0.023 - >256		
	Metronidazole	0.032 - >256	0.094	0.25		Metronidazole	0.047 – 0.5		
	Linezolid	0.125 – 3	1	3		Linezolid	0.38 – 1.5		
	Chloramphenicol	0.75 – 6	3	6		Chloramphenicol	1.5 – 3		
	Doxycycline	0.047 – 4	0.125	1		Doxycycline	0.125 – 16		
	Tigecycline	0.016 – 0.38	0.064	0.125		Tigecycline	0.047 – 1.5		

Table 2. Continued.

Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}	Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}
Pn. ivorii (5)	Penicillin G	0.016 – 0.047			*Pn. lacrimalis* (4)	Penicillin G	0.016 – 0.125		
	Amoxicillin/clavulanic acid	0.016 – 0.032				Amoxicillin/clavulanic acid	0.016 – 0.25		
	Cefotetan	0.125 – 1				Cefotetan	0.016 – 0.38		
	Cefoxitin	0.125 – 0.75				Cefoxitin	0.016 – 0.25		
	Ertapenem	0.004 – 0.032				Ertapenem	0.002 – 0.012		
	Meropenem	0.002 – 0.016				Meropenem	0.002 – 0.016		
	Levofloxacin	0.38 – 64				Levofloxacin	3 – 8		
	Moxifloxacin	0.094 – 64				Moxifloxacin	0.002 – 0.38		
	Clindamycin	0.094 – 2				Clindamycin	0.016 – 0.38		
	Metronidazole	0.094 – 0.25				Metronidazole	0.023 – 0.38		
	Linezolid	0.19 – 2				Linezolid	0.19 – 2		
	Chloramphenicol	1 – 3				Chloramphenicol	0.75 – 3		
	Doxycycline	0.064 – 16				Doxycycline	0.125 – 4		
	Tigecycline	0.032 – 0.25				Tigecycline	0.023 – 0.25		
P. anaerobius (4)	Penicillin G	0.064 – 2			*Pn. gorbachii* (4)	Penicillin G	0.016 – 0.19		
	Amoxicillin/clavulanic acid	0.125 – 4				Amoxicillin/clavulanic acid	0.016 – 0.064		
	Cefotetan	0.5 – 24				Cefotetan	0.5 – 1.5		
	Cefoxitin	0.19 – 3				Cefoxitin	0.064 – 0.5		
	Ertapenem	0.032 – 0.75				Ertapenem	0.012 – 0.023		
	Meropenem	0.023 – 1				Meropenem	0.004 – 0.064		
	Levofloxacin	0.38 – 1.5				Levofloxacin	3 – 64		
	Moxifloxacin	0.19 – 0.25				Moxifloxacin	0.19 – 0.5		
	Clindamycin	0.032 – 1				Clindamycin	0.125 – 0.75		
	Metronidazole	0.032 – 0.25				Metronidazole	0.023 – 0.5		
	Linezolid	0.38 – 1.5				Linezolid	0.75 – 1.5		
	Chloramphenicol	1 – 3				Chloramphenicol	2 – 3		
	Doxycycline	0.5 – 4				Doxycycline	0.064 – 0.38		
	Tigecycline	0.064 – 0.125				Tigecycline	0.016 – 0.094		

Table 2. Continued.

Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}	Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}
A. murdochii (3)	Penicillin G	0.016 – 0.75			*A. tetradius* (2)	Penicillin G	0.023 – 0.032		
	Amoxicillin/clavulanic acid	0.032 – 0.25				Amoxicillin/clavulanic acid	0.032 – 0.064		
	Cefotetan	0.75 – 8				Cefotetan	0.25 – 0.5		
	Cefoxitin	0.125 – 1				Cefoxitin	0.19 – 0.38		
	Ertapenem	0.19 – 2				Ertapenem	0.094 – 0.125		
	Meropenem	0.125 – 0.75				Meropenem	0.094 – 0.125		
	Levofloxacin	1.5 – 4				Levofloxacin	2 – 3		
	Moxifloxacin	0.25				Moxifloxacin	0.19 – 0.38		
	Clindamycin	0.016 – 0.5				Clindamycin	1 – 4		
	Metronidazole	0.19 – 0.5				Metronidazole	0.25 – 0.75		
	Linezolid	0.38 – 0.75				Linezolid	1 – 1.5		
	Chloramphenicol	1 – 3				Chloramphenicol	3 – 3		
	Doxycycline	0.25 – 16				Doxycycline	2 – 8		
	Tigecycline	0.047				Tigecycline	0.125 – 0.19		
At. parvulum (4)	Penicillin G	0.094 – 0.25			*Pn. octavius* (1)	Penicillin G	0.125		
	Amoxicillin/clavulanic acid	0.064 – 0.25				Amoxicillin/clavulanic acid	0.064		
	Cefotetan	2 – 8				Cefotetan	0.5		
	Cefoxitin	1.5 – 3				Cefoxitin	0.25		
	Ertapenem	0.032 – 0.19				Ertapenem	0.094		
	Meropenem	0.125 – 0.25				Meropenem	0.094		
	Levofloxacin	0.38 – 0.5				Levofloxacin	4		
	Moxifloxacin	0.19 – 0.38				Moxifloxacin	0.5		
	Clindamycin	1.5 – 6				Clindamycin	0.047		
	Metronidazole	0.19 – 0.5				Metronidazole	0.38		
	Linezolid	0.75 – 2				Linezolid	0.75		
	Chloramphenicol	4 – 16				Chloramphenicol	2		
	Doxycycline	1 – 2				Doxycycline	0.19		
	Tigecycline	0.064 – 0.5				Tigecycline	0.064		

Table 2. Continued.

Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}	Organism (n)[a]	Antibiotic	MIC range	MIC_{50}	MIC_{90}
R. gnavus (1)	Penicillin G	1			GPAC (4)	Penicillin G	0.023 – 0.125		
	Amoxicillin/clavulanic acid	0.19				Amoxicillin/clavulanic acid	0.016 – 0.094		
	Cefotetan	32				Cefotetan	1 – 4		
	Cefoxitin	4				Cefoxitin	0.125 – 1		
	Ertapenem	0.38				Ertapenem	0.006 – 2		
	Meropenem	0.125				Meropenem	0.008 – 0.75		
	Levofloxacin	64				Levofloxacin	0.5 – 2		
	Moxifloxacin	6				Moxifloxacin	0.064 – 0.38		
	Clindamycin	0.38				Clindamycin	0.094 – 0.125		
	Metronidazole	0.094				Metronidazole	0.064 – 0.38		
	Linezolid	2				Linezolid	0.5 – 1		
	Chloramphenicol	3				Chloramphenicol	1.5 – 3		
	Doxycycline	0.25				Doxycycline	0.094 – 1		
	Tigecycline	0.094				Tigecycline	0.023 – 0.19		
A. lactolyticus (1)	Penicillin G	0.125							
	Amoxicillin/clavulanic acid	0.125							
	Cefotetan	2							
	Cefoxitin	0.5							
	Ertapenem	1							
	Meropenem	0.38							
	Levofloxacin	6							
	Moxifloxacin	0.19							
	Clindamycin	0.047							
	Metronidazole	0.25							
	Linezolid	0.38							
	Chloramphenicol	1							
	Doxycycline	0.38							
	Tigecycline	0.094							

[a]Genus abbrevations: *P., Peptostreptococcus*; *A., Anaerococcus*; *Pa., Parvimonas*; *Pn., Peptoniphilus*; *At., Atopobium*; *R., Ruminococcus*.

Table 3. The overall resistance of GPAC against 14 antibiotics[a].

Antibiotic	Range	MIC_{50}	MIC_{90}
Penicillin G	0.016 – 2	0.047	0.19
Amoxicillin/clavulanic acid	0.016 – 4	0.094	0.38
Cefotetan	0.016 – 32	0.75	3
Cefoxitin	0.016 – 4	0.5	2
Ertapenem	0.002 – 2	0.064	0.19
Meropenem	0.002 – 1	0.064	0.25
Levofloxacin	0.094 – 64	0.75	64
Moxifloxacin	0.002 – 64	0.25	1.5
Clindamycin	0.016 - >256	0.38	2
Metronidazole	0.023 - >256	0.19	0.75
Linezolid	0.125 – 6	1.5	3
Chloramphenicol	0.75 – 16	3	8
Doxycycline	0.047 – 24	1	16
Tigecycline	0.016 – 1.5	0.094	0.38

[a] The overall resistance of GPAC (n=115) against various antiobiotics is indicated.

Discussion

Since GPAC can show poor growth we used a McFarland 2 inoculum. The MIC-values obtained with the quality control strain *B. fragilis* ATCC 25285 show that most of these values are within the expected range. Comparison between a McFarland 1 and 2 inoculum using the quality control strain gave the same MIC-value (data not shown). However, 4 of the 10 MIC values obtained for clindamycin were just above the expected range using McFarland 2. Since GPAC show poor growth when compared to *B. fragilis*, this is not expected to affect our set of data. A practical approach is to use a higher McFarland turbidity as recommended by the manufacturer of E-test.

In this study strains were identified genotypically, since phenotypic identification is not always reliable for all species [20]. It is difficult to compare our results with other published resistance data, since authors may use different breakpoints. For example, some did use breakpoints advised by the Clinical and Laboratory Standards Institute (CLSI), while others used those advised by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Therefore, we have chosen to base a difference in susceptibility on the MIC_{50} and MIC_{90} values, instead of the percentage resistant strains. However, the interpretation of our results using CSLI and EUCAST breakpoints are shown in Table 4.

Table 4. . Percentages resistance of GPAC for 14 antibiotics using CLSI and EUCAST breakpoints.

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Penicillin G						
(interpretive criteria)	**0.5**[b]		**2**	**0.25**[c]		**0.5**
F. magna (31)	31			30	1 (3.2 %)	
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	3		1	3		1
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	2	1		2		1
At. parvulum (4)	4			4		
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)		1				1
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		
Amoxicillin-clavulanic acid						
(interpretive criteria)	**4**[b]		**16**	**4**[c]		**8**
F. magna (31)	31			31		
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			3		
At. parvulum (4)	4			4		
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		

Table 4. Continued.

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Cefotetan						
(interpretive criteria)	**16**[b]		**64**	**n.a.**[d]		**n.a.**
F. magna (31)	31					
Pa. micra (27)	27					
Pn. harei (16)	16					
A. vaginalis (8)	8					
Pn. ivorii (5)	5					
P. anaerobius (4)	3		1			
Pn. lacrimalis (4)	4					
Pn. gorbachii (4)	4					
A. murdochii (3)	3					
At. parvulum (4)	4					
A. tetradius (2)	2					
Pn. octavius (1)	1					
R. gnavus (1)			1			
A. lactolyticus (1)	1					
GPAC (4)	4					
Cefoxitin						
(interpretive criteria)	**16**[b]		**64**	**n.a.**		**n.a.**
F. magna (31)	31					
Pa. micra (27)	27					
Pn. harei (16)	16					
A. vaginalis (8)	8					
Pn. ivorii (5)	5					
P. anaerobius (4)	4					
Pn. lacrimalis (4)	4					
Pn. gorbachii (4)	4					
A. murdochii (3)	3					
At. parvulum (4)	4					
A. tetradius (2)	2					
Pn. octavius (1)	1					
R. gnavus (1)	1					
A. lactolyticus (1)	1					
GPAC (4)	4					

Table 4. Continued.

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Ertapenem						
(interpretive criteria)	**4**[b]		**16**	**1**[c]		**1**
F. magna (31)	31			31		
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			2		1
At. parvulum (4)	4			4		
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			3		1
Meropenem						
(interpretive criteria)	**4**[b]		**16**	**2**[c]		**8**
F. magna (31)	31			31		
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			3		
At. parvulum (4)	4			4		
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		

Table 4. Continued

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Moxifloxacin						
(interpretive criteria)	**2**[b]		**8**	**n.a.**		**n.a.**
F. magna (31)	27	1 (3.2 %)	3 (9.7 %)			
Pa. micra (27)	27					
Pn. harei (16)	16					
A. vaginalis (8)	8					
Pn. ivorii (5)	4		1			
P. anaerobius (4)	4					
Pn. lacrimalis (4)	4					
Pn. gorbachii (4)	4					
A. murdochii (3)	3					
At. parvulum (4)	4					
A. tetradius (2)	2					
Pn. octavius (1)	1					
R. gnavus (1)		1				
A. lactolyticus (1)	1					
GPAC (4)	4					
Clindamycin						
(interpretive criteria)	**2**[b]		**8**	**4**[c]		**4**
F. magna (31)	26	3 (9.7 %)	2 (6.5 %)	29		2 (6.5 %)
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	7		1	7		1
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			3		
At. parvulum (4)	2	2		3		1
A. tetradius (2)	1	1		2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		

Table 4. Continued

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Metronidazole						
(interpretive criteria)	**8**[b]		**32**	**4**[c]		**4**
F. magna (31)	31			31		
Pa. micra (27)	26		1 (3.7 %)	26		1 (3.7 %)
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			3		
At. parvulum (4)	4			4		
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		
Chloramphenicol						
(interpretive criteria)	**8**[b]		**32**	**8**[c]		**8**
F. magna (31)	28	3 (9.7 %)		28		3 (9.7 %)
Pa. micra (27)	27			27		
Pn. harei (16)	16			16		
A. vaginalis (8)	8			8		
Pn. ivorii (5)	5			5		
P. anaerobius (4)	4			4		
Pn. lacrimalis (4)	4			4		
Pn. gorbachii (4)	4			4		
A. murdochii (3)	3			3		
At. parvulum (4)	2	2		2		2
A. tetradius (2)	2			2		
Pn. octavius (1)	1			1		
R. gnavus (1)	1			1		
A. lactolyticus (1)	1			1		
GPAC (4)	4			4		

Table 4. Continued.

	CLSI			EUCAST		
	≤S (n)	I (n [%][a])	≥R (n [%])	≤S (n)	I (n [%])	>R (n [%])
Tigecycline						
(interpretive criteria)	**4**[b]		**16**	**n.a.**		**n.a.**
F. magna (31)	31					
Pa. micra (27)	27					
Pn. harei (16)	16					
A. vaginalis (8)	8					
Pn. ivorii (5)	5					
P. anaerobius (4)	4					
Pn. lacrimalis (4)	4					
Pn. gorbachii (4)	4					
A. murdochii (3)	3					
At. parvulum (4)	4					
A. tetradius (2)	2					
Pn. octavius (1)	1					
R. gnavus (1)	1					
A. lactolyticus (1)	1					
GPAC (4)	4					

[a] The percentage of intermediair/resistent strains is only given for species of which more than 10 strains were present.

[b] Breakpoints for anaerobic bacteria.

[c] Breakpoints for gram-positive anaerobic bacteria.

[d] n.a. = not applicable

The clinically most important GPAC in our study are *F. magna*, *Pa. micra*, and *Pn. harei*. Especially the latter can be difficult to identify phenotypically, since its biochemical features resemble those of *Pn. asaccharolyticus* [10]. In the past *Pn. harei* was probably often misidentified as *Pn. asaccharolyticus*, resulting in limited susceptibility data on this species. Brazier et al. [4] included 44 clinical isolates of *Pn. harei* in a European study, all phenotypically identified. No resistance was reported. In a susceptibility study in England and Wales [5] 4 clinical isolates of *Pn. harei* were included, also phenotypically identified. Resistance (MIC > 256) was reported to clindamycin. In our study, the MIC_{50} and MIC_{90} for clindamycin were 0.25 and 1.5, respectively. The latter being the highest MIC found for *Pn. harei*.

Our study is the first to include *Pn. gorbachii* and *A. murdochii*, although the numbers are low. It is worth to mention that one strain of *A. murdochii* had high

MIC-values for 4 of the 14 antibiotics; doxycycline, ertapenem, levofloxacin, and penicillin G.

Differences in susceptibility to antibiotics were described for *P. anaerobius* and *P. stomatis* [12]. *P. anaerobius* has higher MIC values for amoxicillin, amoxicillin-clavulanic acid, cefoxitin, ertapenem, azithromycin, clindamycin, metronidazole and moxifloxacin than *P. stomatis*, only the MIC_{90} of azithromycin and moxifloxacin was not two dilution steps higher. Brazier et al. [5], also suggests that some GPAC species are more resistant to antibiotics than others. For example, *P. anaerobius* had a higher MIC_{50} for tetracycline, but had lower MIC values for erythromycine as *F. magna*. Roberts et al. [17] described that *P. anaerobius* has higher MIC_{50} and MIC_{90} values for amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, cefotetan and meropenem when compared to *F. magna*, *Pa. micra* and *Pn. asaccharolyticus*. Koeth et al. [11] showed that *F. magna* has a higher MIC_{50} for clindamycin as *Pa. micra* and *P. anaerobius*, while *P. anaerobius* has the highest MIC_{90} for amoxicillin-clavulanic acid.

Metronidazole is often the drug used for empiric treatment of anaerobic infections. However, GPAC strains are described which are resistant to this drug [11, 13, 16]. We encountered one strain of *Pa. micra* which was resistant to metronidazole (MIC > 256). Microbiologists should be aware of this possibility. It is remarkable to notice the difference in susceptibility to the different antibiotics between the three most clinically important GPAC; *F. magna*, *Pa. micra*, and *Pn. harei*. Therefore, it is important to identify clinical isolates of GPAC. *F. magna* and *Pa. micra* can be reliable phenotypically identified using a commercially available enzymatic kit like Rapid ID 32A [20]. However, *Pn. harei* cannot be phenotypically distinguished from *Pn. asaccharolyticus* [10, 20]. The combination of diminished antimicrobial susceptibility, its prevalence and the described virulence factors [8], gives *F. magna* a special position among the GPAC.

Acknowledgement

We are grateful to AB Biodisk for providing the E-test strips.

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Chapter 8

Finegoldia magna

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1. Introduction

1.1 Classification, morphology and epidemiology

Classification. The genus *Finegoldia* belongs to the class of clostridia, phylum Firmicutes. *Finegoldia magna*, the only species present in this genus, is the most commonly isolated gram-positive anaerobic coccus (GPAC) from human clinical specimens [44]. The taxonomy of this species has changed dramatically. In 1933 *F. magna* was first described by Prevot [52] and named *Diplococcus magna* based on the cellular appearance. Holdeman and Moore [25] added the species to the genus *Peptococcus*. In 1983 Ezaki et al. [15] transferred *Peptococcus magnus* to the genus *Peptostreptococcus*. This transfer was based on DNA base composition, DNA-DNA hybridization data and cellular fatty acid profiles. Classification of GPAC using pyrolysis mass spectrometry (PMS) showed that *P. magnus* was distinct from other GPAC species [41]. In addition, 16S ribosomal sequence analyses indicated that *Peptostreptococcus magnus* is phylogenetically distant from the other GPAC. Therefore, the genus *Finegoldia* was proposed to cover the species *F. magna* [43].

Morphology. Cells of different strains of *F. magna* can vary in size. Gram-staining shows clumps of cells, mostly in tetrad formation, with a diameter of 0.8 to 1.6 µm, which are stained gram-positive. A typical clump consists out of large cocci in the middle, surrounded by cells smaller in size. The largest cells have the strongest color, while smaller cells can be decolorized [42].

After 48 hours of incubation on blood agar plates, colonies are approximately 1 mm in diameter. After 5 days of incubation > 90 % of the strains had colonies > 2 mm in diameter. On one plate the colonies can vary in size and color. Some colonies are convex and whitish, while others are flat and translucent. This might give the impression that it is a mixed culture. However, colonies always have a perfect round shape, and are usually slightly convex [42].

Epidemiology. *F. magna* is one of the most frequently recovered anaerobes from clinical infectious material. In such infections approximately 30 % of the anaerobes are GPAC of which around 30 % is *F. magna* [69]. Remarkably, *F. magna* can be isolated from clinical specimens in pure culture and can be surpassed by *Bacteroides fragilis* in this aspect only.

Neut et al. [47] analyzed the presence of GPAC in normal oral, fecal and vaginal microbiota. *F. magna* was found in the vaginal and fecal microbiota (especially healthy adults), but not in the oral microbiota. Riggio et al. [54] determined the presence of *F. magna* in oral samples using a specific PCR. In total, 33 subgingival plaque samples of patients with adult periodontal disease and 60 pus aspirates from patients with acute dentoalveolar abscesses were analyzed.

In 2 subgingival plaque samples *F. magna* DNA was encountered. These results show that *F. magna* is not a major pathogen in adult periodontal disease and dentoalveolar abscesses. Gao et al. [19] analyzed the superficial skin bacterial biota of human forearm of 6 subjects. A total of 91 genera were found, of which 6 were observed in all subjects. One of these was *Finegoldia* AB109769, suggesting that *F. magna* is part of the normal skin microbiota. Higaki et al. [24] analyzed the anaerobes isolated from infectious skin diseases. The most commonly isolated anaerobes were GPAC (66 of 106 strains, 62 %), among these *F. magna* was the most frequently GPAC isolated (27 strains, 41 %). *F. magna* shows similar pathogenic features as *Staphylococcus aureus*. In patients with infected breast abscesses, *F. magna* can be isolated alone or together with *S. aureus* [24]. Since both organisms display synergism of pathogenicity, it is more difficult to cure such infections.

Brook [9] evaluated the recovery of anaerobes, among them *F. magna*, from clinical specimens during a twelve year period. Of all GPAC isolated, 18.4 % were identified as *F. magna*. The majority of these *F. magna* strains (65.7 %) were isolated from abscesses, obstetrical and gynecological infections, and wounds. The highest frequency of recovery of *F. magna* was shown in bone and chest infections. In pediatric patients 680 GPAC were recovered from 598 clinical specimens, from 554 patients [8]. From all these strains, 10.9 % were *F. magna*. The majority was isolated from abscesses. Bourgault et al. [4] evaluated the clinical significance of *F. magna* in 222 patients. Of these patients 183 had an infection in which *F. magna* played an important role. In 17.5 % of these cases *F. magna* was isolated as a pure culture, from infections of bone and joint (56.3 %), soft tissue (37.5 %) and vascular (6.3 %). In mixed infections in which *F. magna* involved, the most frequently isolated facultative bacteria were: group D *Streptococcus*, *Staphylococcus epidermis*, *Escherichia coli* and *S. aureus*. The most frequently found anaerobic bacteria were: *Prevotella melaninogenica*, *B. fragilis* and *Bacteroides* sp. These mixed infections were mainly infections of soft tissue (37.7 %), bone and joint (21.2 %) and foot ulcers (19.2 %). From this data it can be concluded that there is a strong association between *F. magna* found in pure culture and orthopedic procedures and post-operative wound infection.

1.2 Clinical features and pathogenesis

F. magna is capable of producing several virulence factors (Table 1). In 1984, Brook et al. [7] examined the pathogenicity of GPAC in mixed infections. Abscesses caused by two organisms, including one strain of GPAC, were larger compared with abscesses caused by one organism. From their experiments it was concluded that *F. magna* and *Peptostreptococcus anaerobius* were equally

Table 1. Virulence factors of *F. magna.*

Virulence factor	Function	Reference
Collagenase	Breakdown of collagen	32
PAB	Binding to human serum albumin	46
Protein L	Immunoglobulin (Ig)-binding protein	3
	Release of de novo-synthesized mediators	51
SufA	Degradation of fibrinogen	28
	Degradation of antibacterial peptides LL37 and MIG/CXCL9	28, 29
	Release of FAF from bacterial cell wall	29
FAF	Mediation of bacterial aggregation through protein-protein interactions between FAF molecules on neighbouring *F. magna* bacteria	18
	Binding with BM-40, a non-collagenous glycoprotein, present on the skin	
	Blocking the activity of LL-37, an antibacterial peptide	
	Inactivation of the antibacterial peptide MIG/CXCL9	29

important or more important than the other bacteria in mixed infections. This supports the hypothesis that bacteria in mixed infections may have a synergistic nature. The collagenase production of *F. magna* is associated with the site of infection [32]. Collagen is abundantly present in the skin, tendons, and cartilage and is an organic component of bones, teeth, and the cornea. The breakdown of collagen will result in loss of tissue integrity and disease progression, hereby providing an environment suitable for growth of anaerobic bacteria. The production of collagenase may also be important for the growth of asaccharolytic bacteria such as *F. magna*, since during collagen breakdown amino acids are released which may be necessary for growth and survival [23]. Ng et al. [49] determined the aminopeptidase activities of some GPAC strains. *F. magna* together with *Parvimonas* (*Pa.*) *micra* were found to degrade most substrates. There was a correlation between gelatin hydrolysis and the number of aminopeptidases produced. The authors state that gelatin hydrolysis reflects the pathogenetic potential of a strain. The growth of the bacteria can be correlated to the amount of aminopeptidases produced, due to protein degradation.

Myhre [46] described that 42% of the *F. magna* strains is able to bind human serum albumin (HSA). This ability was originally described for different streptococcal species. In group C and G streptococci this is mediated by protein G, and in group A streptococci by protein M [45]. De Chateau et al. [11] demonstrated that some strains of *F. magna* express protein peptostreptococcal albumin binding (PAB) on their cell surface. Sequence analyses revealed homology with the HSA-binding domain of protein G and to the framework regions of protein L (described later). This suggests an interspecies exchange of an HSA-binding protein module.

In general, host binding cell wall proteins of gram-positive bacteria share a common structure, including a (from the distal NH_2 terminus) [56]:
- signal sequence
- variable NH_2-terminal region
- varying number of repeated domains that independently bind different plasma proteins
- proline-rich region supposedly intercalating the protein in the gram-positive cell wall
- COOH-terminal cell wall sorting signal, required to anchor the protein to the cell wall

PAB contains a GA module (protein G-related albumin binding module). This is a centrally located domain of 45 amino acid residues, which is responsible for the binding of HSA. This domain is subject to module shifting. The predecessor of the PAB protein is urPAB. This protein does not contain the shuffled GA module, but has a uGA domain in the NH_2 terminal region. This domain shows 38 % similarity with the GA module and binds HSA to a lesser extent. PAB also contains an analogous uGA domain, which indicates a second binding site for HSA. The affinity for HSA differs between the GA modules. A reason for bacteria to acquire the GA module is that the older uGA domain has lost its function due to the difference in affinity for HSA. The binding affinity for HSA is not only found on the cell surface, but also in the culture supernatant. The growth of HSA-binding strains is stimulated by the addition of HSA to the growth medium [12]. This selective advantage increases the virulence of HSA-binding *F. magna* strains.

Felten et al. [17] studied the binding of 14 *F. magna* strains isolated from bone joint infections to collagen fibrinogen, and fibronectin after implantation of a foreign body. From these strains, 81 % bound to collagen, 69 % to fibrinogen, and 46 % to fibronectin. When these results were compared to the binding abilities from *F. magna* strains from other infections, a correlation was found between fibrinogen binding and bone joint infections (69 % against 20 %). Krepel et al. [33] tried to elucidate the role of *F. magna* in three different polymicrobial environments: intra-abdominal infections, non-puerperal breast abscesses and diabetic foot ulcers. An association was made between phenotypic characteristics and the site of infection. In total 336 clinical specimens were examined, 222 intra-abdominal from which 11 *F. magna* strains were isolated, 58 nonpuerperal breast abscesses from which 21 *F. magna* strains were isolated and 56 diabetic foot ulcers from which 18 *F. magna* strains were isolated. From the *F. magna* strains the hippurate hydrolase, collagenase and gelatinase production was determined. Strains with the lowest enzymatic activity were isolated from intra-abdominal infections. The most

proteolytic strains were predominantly isolated from soft tissue infections. These are the kind of infections which tend to be chronic and healing slowly. Edmiston et al. [14] showed that *F. magna* is the most common anaerobe isolated from non-puerperal breast infections. *F. magna* strains isolated from non-puerperal breast abscesses and diabetic foot infections were shown to have a higher collagenase production compared to *F. magna* strains isolated from intra-abdominal infections [32]. Stephens et al. [62] determined the impact of the presence of GPAC present in deep tissues of chronic wounds. Clinical samples of 18 patients with chronic venous leg ulcers were cultured. Six of these patients had *F. magna*. None of these *F. magna* strains had any hydrolytic enzyme activity or affected the endothelial cell proliferation. All inhibited fibroblast proliferation and keratinocyte wound repopulation.

Björk [3] was the first to describe a novel bacterial cell wall protein which is able to bind with Ig light chains (L chains), therefore this protein was named protein L. L chains are shared between the different Ig classes. Protein L was found to have affinity with IgG, IgM, IgA, $F(ab')_2$, Fab fragments, and with κ and λ L chains. The reaction with λ L chains is very weak compared with κ L chains. Nilson et al. [50] described that protein L binds exclusively to the V_L domain of Ig and not to the C_L domain. This binding strongly depends on the three-dimensional structure of the V_L domain, indicating that several sites of V_L are involved. It requires the spatial proximity of the κI, κIII and κIV light chain molecules. κ L chains represent 65 % of human immonoglobulins, and of the entire κ chain population, κI, κIII and κIV proteins represent 60, 28, and 2 %, respectively [58]. Protein L has five highly homologous domains which are involved in the binding of Ig. These domains interact with the framework regions of the V_L domain [67]. The strength of the binding of protein L with κ chains is less when compared with the binding of the complete Ig. The conformation resulting from the interaction between heavy and light chains in the Ig gives a more favorable binding site for protein L [1]. The binding site of Ig is close to the antigen-binding site, but the interaction between protein L and Ig was not obstructed by occupation of the antigen-binding site. [1, 26]. Åkerström et al. [1] showed that protein L has no disulfide bond or a subunit structure, and that protein L has two non-Ig-binding fragments which were found to be unique. This was confirmed by Graille et al. [22]. A single protein L domain can react with the variable regions of κ L chains of two Fab molecules, in a sandwich fashion. The contact residues in the variable region are remote from the hypervariable loops. It was suggested that the two binding sites on protein L have a different affinity for Ig. In vivo experiments by Smith et al. [57] showed that protein L prefers to target B cells. This is due to the interaction with Ig on the surface of these cells. This interaction strongly activates the B cells which results in

an upregulation of MHC-II and CD86. These surface molecules are important in initiating an antibody response. No specific binding of protein L with other splenocytes, like T cells and certain dendritic cell subsets, was observed. The activation of B cells also results in an increased expression of the target immunoglobulin. When mature B cells are exposed to protein L, a reduction of splenic marginal zone B cells and peritoneal B1 cells was observed [66]. These two B cell subsets are involved in the first-line immune response against foreign invaders. They have a high antigen presenting capacity and secrete preferentially potentially protective natural IgM. B1 cells are located in the cavities of the body and important in contributing to the production of natural antibodies and T cell independent immune responses. Marginal zone B cells are located at the periphery of the splenic periarteriolar lymphoid sheath at the border of white and red pulp, and they are the first to encounter blood born antigens.

The overall design of protein L is similar to that of protein A from staphylococci and protein G from streptococci, but the primary structure is different. When the amino acid compositions of these proteins are compared, protein L has a higher amount of glycine and a lower amount of lysine. No amino acid sequence homology was demonstrated between these three proteins, apart from the carboxyl-terminal transmembrane region. Some similarity was seen between the W-region of protein G and the amino acid sequence of one of the tryptic peptides of protein L. This W-region is proposed to anchor protein G to the cell wall. Protein L is much smaller in size than protein G, which facilitates tissue penetration [26]. One common feature between the three proteins is that they all possess multiple copies of Ig-binding domains. In each protein these domains are highly conserved [22].

Since protein L is able to bind with all human Ig, it is also able to bind with the κ L chain of IgE. Since the binding of anti-IgE with the Fc portion of IgE stimulates the release of histamine from human basophils, it is possible that the binding of protein L with IgE also stimulates the release of histamine, which will trigger an inflammatory response. Histamine and tryptase are both involved in allergic reactions. This stimulation of basophils was described by Patella et al. [51] The release of histamine is dependent on the concentration of protein L. The interaction with IgE present on the surface of basophils mediates the release of protein L. The stimulation by protein L on the basophils is greater than the stimulation by anti-IgE. Patella et al. [51] also described the release of the preformed de novo-synthesized mediators leukotriene C4 (LTC4) from basophils, or PGD2 from human skin mast cells, both chemical mediators of human inflammatory cells. Genovese et al. [20] described the release of histamine from human heart mast cells. They found a significant correlation between histamine release and tryptase release, and the

release of LTC_4. It is interesting to note that mast cells tend to accumulate at the site of a chronic infection [36]. The release of de novo-synthesized mediators may contribute to the pathogenesis of the infecting strain. It is hypothesized that this may cause mycocardial damage in patients with bacterial infections [20]. Protein L helps the bacteria to adhere to the wound surfaces. The covering of the bacterial cell wall by host proteins allows the bacteria to evade the immune response of the host [26].

Protein L is expressed at the surface of ± 10 % of the *F. magna* strains [26, 31]. Some protein L molecules are released into the growth medium, but most molecules are associated with the cell wall. The protein L from the growth medium shows a considerable heterogeneity in size. This indicates proteolytic degradation of the released protein [1]. Kastern et al. [31] found that only *F. magna* strains which express protein L possess the protein L encoding gene. In non-expressing protein L strains, this gene is not present rather than being down-regulated. The features described for protein L may explain why protein L expressing *F. magna* strains are more often associated with clinical infections than non-expressing strains.

Kastern et al. [31] determined the presence of protein L in 30 *F. magna* strains, all derived from clinical specimens. Four of these strains expressed protein L, and all of these four strains were isolated from women with bacterial vaginosis. The negative strains were from healthy women (n=19), men (n=4) and women with bacterial vaginosis (n=3). These results indicate that there is a correlation between protein L expressing *F. magna* strains and bacterial vaginosis. This was confirmed by de Château et al. [12]. They determined the presence of protein L and HSA-binding protein in 48 *F. magna* strains. Thirty of these strains were isolated from suppurative infections. One of them expressed protein L and 16 strains (53 %) were binding HSA. Eight strains of the 48 strains were isolated from patients with bacterial vaginosis. None of these strains showed HSA binding and five were expressing protein L. The remaining ten strains of the originally 48 were commensal strains, and none of them was expressing protein L or binding HSA. These results confirm the correlation between protein L expressing *F. magna* strains and bacterial vaginosis. Furthermore, it also shows that *F. magna* strains isolated from localized suppurative infections preferentially express HSA binding protein. It is striking to notice that no strains were found which expressed protein L and HSA-binding protein in combination. This was also noticed by Ng et al. [48]. A total of 32 *F. magna* strains, from different origins and countries, were analyzed. Strains were found to be protein L expressing, HSA-binding protein expressing, or expressing neither of them. No strains were found to express both proteins.

Molecular typing of these strains showed that protein L and HSA-binding strains are associated with genotypic clusters.

Recently two other proteins which enhance the virulence of *F. magna* have been described, i.e. a subtilisin-like proteinase (SufA) and a *F. magna* adhesion factor (FAF). FAF is expressed by more than 90% of the *F. magna* strains. This protein is cell wall bound and can be released in the growth medium. The soluble form of FAF causes large protein aggregates and the cell wall bound FAF induces bacterial aggregation through protein-protein interactions between the FAF proteins of the different *F. magna* cells [18]. FAF has the typical features of surface proteins of gram-positive bacteria; a C-terminal part with a cell wall spanning region, a membrane anchor and an intracellular charged tail. *F. magna* strains which express FAF interact with BM40 and colonize the skin in the same way as SufA expressing *F. magna* strains. Another feature of FAF is that it protects *F. magna* against LL-37, an antimicrobial peptide [18]. BM40 is able to stimulate wound healing and it increases the albumin transport across the endothelium. The increase of albumin will stimulate PAB expressing *F. magna* strains in their growth.

SufA is the first described proteinase for *F. magna* [28]. It is associated with the cell wall, but is also released in the growth medium. Most *F. magna* strains possess homologs of SufA. SufA is able to degrade the antibacterial peptides LL-37 and MIG/CXCL9. Hereby *F. magna* enhances its own growth and can spread to commensal areas where it is not present under normal conditions. Since SufA is a subtilisin, it first has to undergo auto-catalytic maturation before it can be active. For full enzymatic activity, dimer formation of SufA is required. The antibacterial peptide MIG/CXCL9 binds with the CXCR3 receptor which activates the G-protein [35]. This receptor is expressed on eosinophils, NK cells, activated T cells, and endothelial cells. Remarkably, MIG/CXCL9 degraded by SufA is still able to bind to the CXCR3 receptor [29]. The fragments of MIG/CXCL9 are still able to kill *Streptococcus pyogenes*, but *F. magna* remains unaffected. This may be explained by the fact that the dimer formation is affected due to the fragmentation of MIG/CXCL9. This may result in a reduced antibacterial activity against *F. magna*. During infections caused by *S. pyogenes* an ecological niche can be created for *F. magna*. SufA is also able to release the FAF protein from the cell wall of *F. magna*. Karlsson et al. [29] described that FAF is able to bind with MIG/CXCL9 with a high affinity. The release of FAF from the cell wall results in a decrease of the antibacterial activity of MIG/CXCL9, hereby promoting the growth of *F. magna* during inflammation. In human plasma SufA degrades fibrinogen, a major clotting enzyme [30]. It increases the thrombin-induced coagulation time in a dose-dependent manner. Fibrinogen is cleaved by thrombin to create fibrin, which forms a temporary matrix in which cells can proliferate during wound repair. In its soluble

form SufA forms dimers and/or multimers, which are proteolytically more active when compared with the monomers [30]. Fibrinogen consists out of 3 pairs of non-identical chains Aα, Bβ and γ [40]. Firstly, SufA removes the C-terminal portion of fibrinogen Aα chains. Secondary, the NH_2-terminal part of the Bβ chains are attacked. At higher concentrations of SufA the Aα chains are further processed. Hereby, the central polymerization sites are removed. SufA associated with the cell wall prevents the formation of fibrin networks by binding to keratinocytes [30]. When the skin is damaged or infected, *F. magna* SufA expressing bacterial cells will be in contact with plasma proteins. The fibrinogen present in the plasma will be broken down by SufA present on the cell wall. The formation of a fibrin network is delayed. The fibrinopeptides (FPA and FPB) which are released during the cleavage of fibrinogen are chemotactic agents for neurophils, macrophages and fibroblasts. They also exert antibacterial properties against gram-negative and gram-positive bacteria [63]. It seems that besides the clotting also other fibrinogen mediated processes are disturbed by SufA. FAF and SufA expressing strains might impair wound healing, as has been described by Stephens et al. [62].

1.3 Genome organization

Recently, the genome of *F. magna* ATCC29328 was assessed by Goto et al. [21]. It consists of a circular chromosome (1.797.577 bp, average G+C content 32.3 %) and a plasmid pPEP1 (189.163 bp, average G+C content 29.7 %). Complete gene sets for the biosynthesis of glycine, serine, threonine, aspartate, and asparagine were present. There were no carbohydrate phosphotransferase system (PTS) genes present for glucose, maltose, mannose, glucitol, cellobiose, and lactose. PTS genes for mannitol, galactitol, and sucrose were incomplete. A lot of genes encoding aminoacid/oligopeptide transporters were found on the genome. This enables *F. magna* to take up amino acids from the environment for growth and survival. Genes for superoxide reductase, NADH oxidase, and putative NADH dehydrogenase were also present. They probably are important for the survival of *F. magna* in intermediate aerobic conditions, such as mucosa and the skin. Virulence factors for antiphagocytosis were encoded by genes present on the chromosome and the plasmid, one on each. In total 4 genes encoding albumin-binding protein homologs were present, 3 on the chromosome and 1 on the plasmid. In total, 10 genes encoding collagen binding proteins were found, 5 on the chromosome and 5 on the plasmid. In total 20 genes encoding N-acetylmuramoyl-L-alanine amidase homologs (Cwp66) were encountered, most of them located on the chromosome. These proteins play a role in the adherence of bacteria to host cells. The presence of genes encoding sortase was assessed on the chromosome and plasmid. On the chromosome 4 genes encoding sortase homologs were

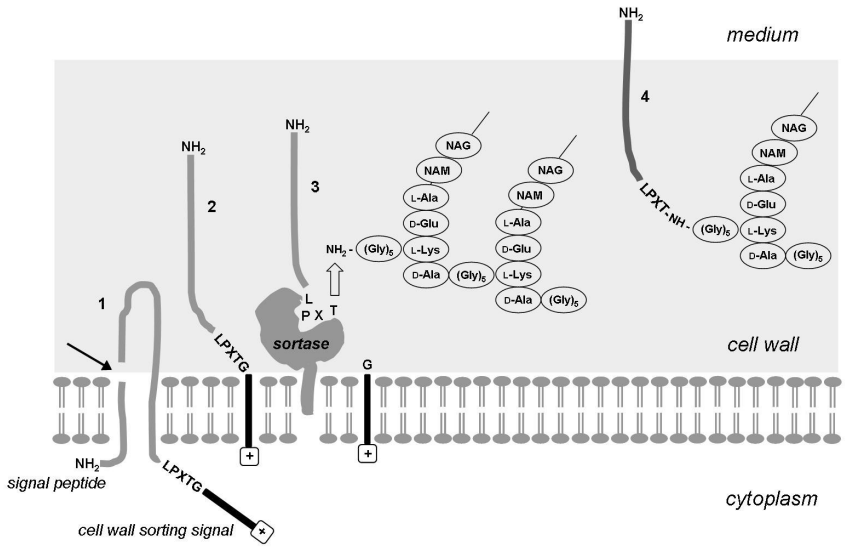

Fig. 1 A schematic overview of the mechanism of covalent binding of surface proteins. (Adapted from: Cossart, P. and Jonquières, R., *Proc. Natl. Acad. Sci. USA,* 97, 5013, 2000)

1. The precursor protein is directed by the N-terminal region of the signal peptide to the cell membrane, after which it is cleaved by a signal peptidase (arrow).
2. The precursor retains within the secretory pathway, probably due to the positively charged tail and the hydrophobic region.
3. The pentapeptide motif LPXTG is cleaved by sortase between the threonine and glycine residues. An amide-bond is formed between the produced carboxyl and peptidoglycan in the cell wall.
4. The surface protein is anchored in the cell wall.

present and on the plasmid 7. Especially the presence of 7 sortase homologs on the plasmid is interesting. It is the highest number of sortase homologs present on a plasmid, for as far as genome sequences are determined for gram-positive bacteria. This feature might be unique for *F. magna*. Since plasmids are considered to be of foreign origin, the amount of sortase homologs present on a plasmid may play an important role in the pathogenesis of *F. magna*. Sortases, are extracellular transpeptidases, which catalyze the cell anchoring of cell wall proteins. Sortases can be grouped into 4 or 5 different classes [10, 13]. Each subgroup has his own preference for substrates, depending on the amino acids present in the cell wall sorting signal pentapeptide motif. Sortase A is the most important one and catalyzes the highest number of substrates. The precursor of a cell wall bound protein is synthesized in the cytoplasm with an N-terminal signal

peptide and a C-terminal sorting signal (Figure 1). The cell wall sorting signal consists out of a pentapeptide motif (for sortase A: LPXGT motif) a hydrophobic region, and a tail of charged residues [55]. The N-terminal signal peptide directs the precursor to the membrane for translocation. It is assumed that, after cleavage by a signal peptidase, the hydrophobic region and the positively charged tail retain the precursor within the secretory pathway, until the sortase has recognized the substrate [56]. The membrane-anchored sortase A cleaves between the threonine and glycine residues of the LPXTG-like motif [38]. An amide-bond is formed between the carboxyl group produced by the cleavage of the LPXGT motif and peptidoglycan of the cell wall. Since most virulence factors are displayed on the cell wall, sortases play an important role in the virulence of bacteria. The amount of cell wall anchored protein will be enriched and more varied. This may result in an enhancement of interaction between host tissue and other bacteria in mixed infections. Therefore, sortases are a possible target for the development of new therapeutic drugs against bacterial infections [37].

1.4 Diagnosis

Phenotypical techniques. F. magna strains are difficult to identify, since the strains do not show any saccharolytic activity and only produce acetate as volatile fatty acid (VFA). Identification is therefore based on negative reactions. Microscopic appearance may be rather variable and is therefore not suitable for identification. Ezaki et al. [16] showed that GPAC can easily be identified by their amidase and oligopeptidase activies. Murdoch et al. [42] characterized the described species more fully, among them *F. magna*. In total 9 reference strains of *F. magna* and 78 clinical isolates were analyzed. All strains showed similar proteolytic activity. Strongly positive reactions were obtained for: arginine, leucine, and pyroglutamyl aminopeptidase. Negative reactions were obtained for: proline, phenylalanine, and glutamylglutamyl aminopeptidases. Variations were observed for other proteolytic enzymes. No acid production from mannose, raffinose, glucose, and trehalose was found. A minor part of the strains (22 %) was able to produce a small amount of acid from fructose. Most of the strains were negative for alkaline phosphatase, but 7 % was found to be positive, including the type strain. 19 % of the strains were able to produce catalase. All strains were resistant to sodium polyanethol sulfonate (SPS). If a metronidazole disk is used to exclude anaerobic staphylococci or capnophilic streptococci, it should be noted that *F. magna* is able to produce colonies in the inhibition zone after 48 hours of incubation. Recently, Song et al. [59] developed a flow chart which made the identification of GPAC easier. In this flow chart *Pa. micra* and *F. magna* are differentiated from the other GPAC by their inability to produce β-glucuronidase,

and to ferment glucose and their ability to produce pyroglutamyl arylamidase. *Pa. micra* and *F. magna* are differentiated from each other by the production of proline arylamidase, *Pa. micra* is a producer and *F. magna* is not [59].

Two current methods to phenotypically identify anaerobes are the Vitek system and Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectometry (MALDITOF-MS). Recently, BioMérieux (Marcy, France) has developed a new colorimetric identification card (ANC card), which can be used in the Vitek 2 system, for the identification of anaerobes, including *F. magna*. Evaluation of this card showed that *F. magna* can be reliably identified using this method [39, 53]. MALDITOF-MS is a promising new method to identify bacteria, however no evaluation studies have been published yet, which describe the suitability of the method for the identification of GPAC, including *F. magna*.

Genotypical techniques. Nowadays, nucleic acid based techniques are available to improve the identification of bacteria. Song et al. [60] evaluated if 16S rDNA sequencing is suitable for the identification of GPAC. They established that the quality of the sequences in the public databases can be poor and might lead to misidentification. They sequenced the type strain of *F. magna* and 36 clinical isolates. When the sequences of the clinical isolates were compared with the sequenced type strain, the homology was >99 %. However, when they were compared with the sequences available in the public databases homology was <98 %. When analyzing sequences of GPAC it is best to compare them with the sequences published by Song et al. [60].

PCR methods to identify *F. magna* were developed by Song et al. [61] and by Riggio et al. [54]. Primers specific for *F. magna* were designed and validated. Song et al. [61] validated the primer set on the type strain of *F. magna* and 60 sequenced clinical isolates. The primers were shown to be specific. Riggio et al. [54] validated the specificity of their primers with 5 *F. magna* strains. Furthermore, they applied their PCR method on subgingival plaque samples from patients with adult periodontal disease and on 60 pus aspirates from subjects with acute dentoalveolar abscesses. No nonspecific reactions were encountered.

A species-specific 16S rRNA based probe was developed by Wildeboer-Veloo et al. [68]. The fluorescently labeled probe was used to rapidly and reliably identify *F. magna*, using fluorescence *in situ* hybridization (FISH). The probe was designed and validated using sequences of reference strains of *F. magna* and 26 clinical isolates and was shown to be specific. The probe was applied on 100 unknown clinical isolates of GPAC, of which 29 strains were identified as *F. magna*. Permeabilization of the cell wall of *F. magna* strains, using proteinase-K, was

necessary prior to hybridization. The probe has the potential to be suitable for direct application on clinical material.

2. Methods

2.1 Sample preparation

No special sample preparation is necessary for the isolation of *F. magna.* The usual sample preparation conditions for the isolation of anaerobic bacteria will be sufficient [27]. Clinical specimens should be collected, avoiding contamination with commensal skin or mucus surface microbiota. Transportation of specimens should take place in an appropriate transport medium for anaerobic bacteria.There is no selective medium available for the isolation of *F. magna*. *F. magna*, like most GPAC, will grow on a standard medium, like Brucella Blood Agar (BBA) and Schaedler blood agar. Mostly, clinical specimens suspected to contain anaerobic bacteria are cultured on a set of plates. A universal medium (for example BBA), phenyl alcohol blood agar (PEA), which prevents swarming of bacteria, and media selective for gram-negative anaerobes like Bacteroides bile esculin agar (BBE) and kanamycin vancomycin laked blood agar (KVLB). The latter promotes also the pigmentation of bacteria. *F. magna* will grow on the universal medium and PEA [27]. Strains suspected to be *F. magna*, after being tested for aerotolerance, can be identified as described in section 1.3.

2.2 Detection procedures

F. magna can be recognized by its colony form and cell morphology, as described above. However, this is not sufficient for identification. Other biochemical features which can be used are its slow growth, the odor, resistance to SPS, nitrate reduction, coagulase production, no indole production, and enzyme profile [27, 34, 59].

Bassetti et al. [2] reported a case of endocarditis caused by *F. magna*. However, in this case the blood cultures were initially negative. They showed that the detection of *F. magna* in blood depends on the type of blood culture systems used. Bact/ALERT FA and FN bottles were still negative after 4 weeks of incubation. SEPTI-CHEK BHI-S bottles and the ISOLATOR system already showed growth after 2 days of incubation. This may account for the fact that bacteremia caused by *F. magna* is rare [64]. Microbiologists should be aware of the fact that a bacteremia caused by *F. magna* can be missed when the blood culture system Bact/ALERT is used.

We present below three molecular-based techniques for identification and confirmation of *F. magna.*

(i) Protocol of Song et al. [61]

Principle. A multiplex PCR assay was developed to rapidly identify GPAC. Genus- and species-specific primers were used. Since *F. magna* is the only species present in the genus *Finegoldia*, it can be identified directly to species level.

Procedure:

(1) Suspend one or two colonies of the bacterial strain in 50 μl Tris-HCl/EDTA/saline (pH 8.0), and incubate for 10 min at 95 °C.
(2) Centrifuge for 2 min at 18 600 g, and decant the supernatant.
(3) Resuspend the pellet.
(4) Prepare the PCR mixture (50 μl) containing: 1.25 U *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 μl of bacterial lysate, 0.25 μM of the universal primer 1392B and 0.5 μM of the genus/species-specific primer FIGD.
(5) Perform the PCR for 35 cycles; denaturation at 95 °C for 20 s, annealing at 50 °C for 1 min, and extension at 72 °C for 30 s. To the final extension add a cycle of 72 °C for 5 min.
(6) Analyze PCR products on a 2 % agarose gel, followed by ethidium-bromide staining.
(7) Visualize under UV illumination.

Note. *F. magna* strains will yield an amplicon size of 1200 bp.

(ii) Protocol of Riggio et al. [54]

Principle. This PCR assay was developed for the direct detection of *F. magna* in subgingival plaques samples.

Procedure:

(1) Take a subgingival plaque sample using a sterile curette and put it in 0.5 ml freshly prepared fastidious anaerobe broth (Bioconnections). Mix for 30 s.
(2) Add 3 μl achromopeptidase (20 U/μl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to 100 μl of plaque sample. Incubate at 56 °C for 30 min, boil for 5 min and store at -70 °C.
(3) Prepare the PCR mixture (50 μl) containing: 45 μl 1xPCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100), 1 U *Taq* DNA polymerase (Promega), 0.2 mM dNTPs, 0.2 μM of each primer, and 5 μl lysed plaque sample.
(4) Perform the PCR, starting with a denaturation step of 94 °C of 5 min followed by 35 cycles of denaturation of 1 min at 94 °C, annealing of 1 min at 60 °C, and extension of 1 min at 72 °C. To the final extension add a cycle of 72 °C for 10 min.

(5) Analyze the PCR products on a 2 % agarose gel containing ethidium bromide, by adding 2 μl gel loading dye [0.25 % (w/v) bromophenol blue, 50 % (v/v) glycerol, 100 mM EDTA, pH 8.0] to 10 μl PCR product.
(6) Visualize under UV illumination.

Note. *F. magna* strains will yield an amplicon size of 553 bp. A positive and negative control were added to the assay, consisting of genomic *F. magna* DNA and water, respectively. An appropriate DNA ladder should be included during electrophoresis.

(iii) Protocol of Wildeboer-Veloo et al. [68]

Principle. *F. magna* strains are identified by fluorescent *in situ* hybridisation, using a fluorescently labeled species-specific 16S rRNA-based probe.

Procedure:

(1) Take some colonies of bacterial cells and suspend them in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4/L, pH 7.4). Add an equal volume of 96 % v/v ethanol. Mix and store at -20 °C.
(2) Spot the ethanol-fixed cells on three gelatin-coated glass-slides and dry at room temperature.
(3) Immerse the slides in 96 % v/v ethanol for 10 min and allow them to dry.
(4) Add 100 μl proteinase K (500 mg/L in 50 mM Tris-HCl, pH 7.6) to each slide and cover it with a coverslip. Incubate for 10 min at room temperature.
(5) Stop the enzymatic reaction by incubating the slides in 96 % v/v ethanol for 2 min. Allow the slides to dry.
(6) Prepare three different hybridisation mixtures; one for the positive control (probe EUB338), one for the specific probe (Fmag1250) and one for the negative control (probe non-EUB338). Each mixture contains: 10 μl probe (100 ng/μl) and 110 μl hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, SDS 0.1 % w/v).
(7) Add to each slide approx. 100 μl of a probe-hybridisation mixture and cover the slide with a coverslip.
(8) Incubate overnight at 50.0 °C in a dark humid chamber/box.
(9) Wash the slides in washing buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2) for 15 min at 50.0 °C. Rinse the slides quickly in milli-Q and dry with compressed air.
(10) Add approx. 10 μl vectashield® (Vector laboratories, Burlingame, USA) and a coverslip. Fix the coverslip with nailpolish.
(11) Examine the slides under an epifluorescence microscope and compare the signal of the specific probe with the positive and negative control.

Note. The proteinase K treatment is necessary to permeabilize the cell wall of *F. magna* to enable the probe to access the bacterial cell. However, this treatment

also increases loss of bacterial cells from the slide. It is therefore recommended to put sufficient bacterial cells on the slide. This technique has the potential for direct application on clinical material.

3. Conclusion and future perspectives

F. magna is part of the commensal microbiota and clearly an important pathogen. *F. magna* is able to express different virulence factors; collagenase, PAB, protein L, SufA, and FAF. The sequencing of the genome of *F. magna* ATCC29328 revealed a total of 4 albumin-binding proteins, 10 collagen-binding proteins, 20 Cwp66 homologs, and 11 sortase homologs. Beside these proteins, some other putative virulence factors may have been revealed, e.g. hemolysin and bacteriocin. The high number of sortase homologs enables *F. magna* to enrich and vary cell wall anchored proteins, hereby increasing its virulence.

Each of these virulence factors increases the pathogenicity of *F. magna*. Until now, it is unclear how many virulence factors one *F. magna* strain can express at the same time. Especially the high number of sortase homologs enables *F. magna* to acquire several virulence factors in one strain. Ng et al. [48] studied 32 *F. magna* strains and found none of the strains expressed both protein L and HSA-binding protein. Molecular typing of these *F. magna* strains expressing protein L or protein PAB revealed that these strains form their own genotypic cluster. More studies should be performed whether *F. magna* strains expressing certain virulence factors, e.g. collagenase etc. are genotypically different from each other. It is also interesting to know if more expressed virulence factors are associated with genotypic clusters of *F. magna*.

Several authors [11, 12, 14, 17, 32, 62] have shown that there is a relationship between certain virulence factors of *F. magna* and the site of infection. For example, *F. magna* strains isolated from non-puerperal breast abscesses and diabetic foot infections have a higher collagenase production when compared with strains isolated from intra-abdominal infections [32]. This site-specific infection and expression of virulence factors have also been shown for protein L and HSA-binding protein [31]. However, these conclusions are based on a small number of studies, and therefore on a relatively small number of strains. Whether there is a site-specific infection for collagenase, SufA, and FAF expressing *F. magna* strains is still unknown. Further studies are necessary to eludicate all virulence factors of *F. magna* and their role in certain infections.

It is also shown that *F. magna* has a negative influence on healing of chronic wounds [62]. It is not clear whether these strains express only one virulence factor or several, and if there is a coherence between the different virulence factors, as has been described by Karlsson et al. [29] between SufA and FAF. In order to

eludicate the role of *F. magna* and the expression of virulence factors, microbiologists should pay more attention to the identification of *F. magna* from clinical specimens. In general, GPAC are susceptible to the antibiotics used to treat an anaerobic infection [44]. However, microbiologists should realize that *F. magna* has one of the highest resistance rates of the GPAC [6, 65], and that there seem to be geographical differences in resistance [5, 44]. To get a coherent picture of *F. magna* and its pathogenicity, studies with larger groups of strains should be performed. Hereby, studying all the specific virulence factors of *F. magna* at the same time and taking the site of infection into account.

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Chapter 9

Summary and general discussion

Summary and general discussion

In this thesis the biochemical and nucleic acid-based methods which are helpful for the identification of gram-positive anaerobic cocci (GPAC) on genus and species level are studied and evaluated. In the course of these studies a large and well-defined collection of GPAC was created, enabling us to perform in depth studies on the antimicrobial susceptibility of the different species. Insight in the emerging antimicrobial resistance in anaerobic bacterial species, with a focus on GPAC in this thesis, is of importance regarding their clinical relevance. This latter aspect is highlighted by a review on the pathogenicity of *Finegoldia magna*.

Phenotypic identification based on biochemical features

In Chapter 2 clinically relevant GPAC are phenotypically identified using the commercially available Vitek ANC card. The obtained identification was compared with the genotypic identification using 16S rRNA gene sequencing. The database of the Vitek system contains only a limited number of GPAC species, but among them are the two most encountered species *Finegoldia magna* and *Parvimonas* (*Pa.*) *micra*. The third commonly encountered species *Peptoniphilus* (*Pn.*) *harei* is not present in the database and is consistently misidentified as *Peptoniphilus asaccharolyticus.* With the Vitek ANC card 51.9 % of the clinically encountered GPAC strains were identified to the species level and 69.2 % were correctly identified to the genus level. In general, the ANC card shows the same limitations as standard classical phenotypic methods [5], e.g. *Pn. harei* cannot be phenotypically differentiated from *Pn. asaccharolyticus*. Since *Pn. harei* is encountered in clinical material in a similar frequency as *Pa. micra*, the number of correctly identified strains at the species level would increase from 63.5 % to 78.8 %, whenever the system could be able to indicate, which it can't, that the strain is either *Pn. harei* or *Pn. asaccharolyticus*. The only GPAC species present in the VITEK database are *F. magna*, *Pa. micra*, *Peptostreptococcus anaerobius* and *Pn. asaccharolyticus*. Therefore, species belonging to the genus *Peptoniphilus* can be correctly identified at the genus level only. Species belonging to the genus *Anaerococcus* cannot be identified or are misidentified. From this study we concluded that with the ANC card *F. magna* , *Pa. micra* and *P. anaerobius* can be reliably identified but other species of GPAC not.

In Chapter 3, clinically relevant GPAC were phenotypically identified using the enzymatic reactions of the Rapid ID 32A commercial system. Tests with this enzymatic kit yields a code which is entered into a database to obtain an identification. However, this database contains a limited number of species and is often not updated for new species. For example, when we enter the code for *Peptoniphilus ivorii* into the database the obtained identification may be *Clostridium*

difficile. We performed a study using only relevant enzymatic reactions and compared the results with the genotypic identification which was either 16S rRNA sequencing or fluorescent *in situ* hybridization (FISH) using species-specific 16S rRNA-based probes. *F. magna* (29/29) and *Pa. micra* (24/28) can be identified reliably using this phenotypic method of identification. However, *Pn. harei* (n=17) could not be differentiated from *Pn. asaccharolyticus* and 4 of the 17 tested strains were either misidentified or could not be identified. *P. anaerobius* and *Peptococcus niger* were all phenotypically correctly identified. *Pn. ivorii* and *Anaerococcus vaginalis* can be identified correctly using the relevant enzymatic reactions, but were also frequently not identified. Other GPAC species like *Anaerococcus lactolyticus*, *Peptoniphilus lacrimalis, Peptoniphilus octavius* and *Anaerococcus tetradius* could not be identified.

Genotypic identification using 16S rRNA-based oligonucleotide probes and analyses of 16S rRNA sequences

Chapter 3 also describes the development of species-specific 16S rRNA based probes. Sequence results of the 16S rRNA gene of clinical isolates showed for some species a large intra-species variation. Therefore, designing species-specific 16S rRNA probes solely on sequences of reference strains might result in probes which will not hybridize with all strains of the same species. To overcome this problem, species-specific probes were designed not only using sequences of reference strains but also of clinical isolates. Species-specific probes were designed for *F. magna*, *Pa. micra*, *Pn. harei*, *A. lactolyticus*, *A. vaginalis*, *Pn. ivorii*, and *P. anaerobius/stomatis*. The validation of these probes on the reference strains and clinical isolates, of which the sequences were used to design the probes, showed that they were specific. The set of probes was used to identify 100 unknown clinical isolates. If strains could not be identified, the sequence of the 16S rRNA gene was determined to assess whether these were species not detected by the set of probes or were false-negative results. Of the 100 isolates, 87 could be identified using the set of probes. Sequence results of the remaining strains showed that most of them were species not detected by the probes. However, two strains of *A. vaginalis* and one strain of *A. lactolyicus* were negative with their corresponding probe, due to mismatches. Hill et al. [4] demonstrated a large intra-species variation for these two strains. Designing probes for species with such a large intra-species variation might result in false-negative results, as is the case in our study.

Analysis of the sequence data of 71 clinical isolates, for which a near full-length sequence was obtained, showed that 25 isolates had a sequence similarity of <98 % with their closest relative. This indicates that certain species e.g.

A. lactolyticus, *Pn. harei* and *Pn. ivorii* have a large intra-species variation. Therefore these species may not represent single species, but should be studied more in depth to confirm whether differentation in more species is justified or not. After the publication of the study in this Chapter, Song et al. [10] described three new species of GPAC, among them *Peptoniphilus gorbachii* and *Anaerococcus murdochii*. The closest relatives of these two species are *Pn. harei* and *A. lactolyticus,* respectively. Sequence analysis of the strains of *Pn. harei* (n=4) and *A. lactolyticus* (n=7) with a sequence similarity of <98 % showed that one strain and 5 strains were in fact *Pn. gorbachii* and *A. murdochii*, respectively. This also means that the species-specific probe for *Pn. harei* and *A. lactolyticus* will hybridize not only with the species for which they were designed but also with the new species. This shows one of the disadvantages of using species-specific 16S rRNA-based probes for the identification of bacteria. The addition of new species to a genus might cause the probes to be not species-specific anymore. Regularly checking the probes against a public available database as Genbank is recommended. An important advantage of using FISH for the identification of bacteria is that the probes can directly be applied on clinical material.

In Chapter 4, a case of a tongue abscess is described. It clearly shows how FISH aided in the identification of the different species present in the collection of purulent material, aspirated from the abscess. Species detected with FISH could not be isolated by common culture techniques.

The identification of 100 unknown clinical isolates of GPAC using molecular techniques also gave a better insight in the clinical prevalence of certain species. Among the most commonly isolated GPAC from clinical specimens are *F. magna* and *Pa. micra* [12]. This was confirmed in our study. However, *Pn. asaccharolyticus* is also described as being commonly encountered. In our study, no *Pn. asaccharolyticus* strains were encountered. In contrast, 17 % of the GPAC were positive with the species-specific probe for *Pn. harei*. Song et al. [11], used a multiplex PCR assay using genus- and species-specific primers for the identification of GPAC. A total of 190 strains was identified using this multiplex PCR assay, and 25% were shown to be *Pn. harei*. As in our study, no *Pn. asaccharolyticus* strains were encountered. The biochemical features of *Pn. harei* and *Pn. asaccharolyticus* show that these two species cannot be differentiated from each other phenotypically [5]. This clearly shows that *Pn. harei* in the past has been misidentified as *Pn. asaccharolyticus*. As described in Chapter 5, the clinical relevance of *Pn. asaccharolyticus* has been highly overestimated. *Pn. harei* appears to be far more often present in clinical material than perceived before.

Identification using MALDI-TOF MS

Recently another phenotypic technique has gained increasing popularity for the identification of bacteria, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). In Chapter 6 it is shown how we used this technique for the identification of GPAC. A database was constructed using sequenced clinical isolates and reference strains. The obtained database was used for the identification of 107 unknown clinical isolates. The MALDI-TOF MS identification was compared with the genotypic identification which was either 16S rRNA gene sequencing or FISH using the species-specific probes described in Chapter 3. Of these 107 strains, 11 could not be identified of which 5 due to the absence of reference strains/spectra in the database. The remaining 6 strains were genotypically identified as *F. magna* (3 strains), *A. vaginalis* and *Pn. ivorii* (2 strains). For 5 of these strains the sequence similarity with their closest relative could be calculated and 3 strains had a sequence similarity of < 98% (1 *F. magna* strain and 3 *Pn. ivorii* strains). The dendrogram obtained with MALDI-TOF MS showed that all analyzed clinical isolates cluster with their closest relative, except for the 6 strains that could not be identified even though reference strains/spectra were present in the database. Comparing the dendrogram with the phylogenetic tree calculated using the 16S rRNA sequences, showed the same relationships between the different strains. This is probably due to the fact that with MALDI-TOF MS, among other proteins, most of the peaks in the spectra originate from ribosomal proteins [1]. The results of this study show that MALDI-TOF MS is not only a reliable tool for the identification of GPAC, but that it is also suitable for describing the taxonomy of the different species. The 16S rRNA gene sequence analyses of the GPAC strains and the MALDI-TOF MS results both show new not yet described species.

The antibiotic susceptibility of GPAC

In Chapter 7 the difference in antibiotic susceptibility between species of GPAC is described. Fifteen different species against 14 antibiotics were tested. In contrast to other studies the strains in this study were identified genotypically by FISH and 16S rRNA gene sequencing instead by using a phenotypic identification. This is the first antibiotic resistance study in which *Pn. harei* is reliably identified and therefore the first to compare its antibiotic susceptibility with other GPAC. Furthermore, also the first resistance data is given for newly described species as *Pn. gorbachii* and *A. murdochii*. In studies on resistance data often a percentage of resistant strains is given. However, different breakpoints derived from e.g. EUCAST and CSLI guidelines, for the same antibiotic are used. This makes it difficult to compare the results of the different studies. Therefore we have chosen to discuss our resistance

data based on the MIC_{50} and MIC_{90} of the different antibiotics for each species. The MIC_{50} and MIC_{90} were only calculated for species of which more than 10 strains were present in the study. In fact, these were the three most encountered GPAC species *F. magna*, *Pa. micra* and *Pn. harei*. In general, *F. magna* showed the highest resistance (MIC_{50} and MIC_{90}) rates for penicillin G, amoxicillin-clavulanic acid, clindamycin and tigecycline while *Pa. micra* had the lowest resistance rates for levofloxacin, metronidazole and doxycycline. *Pn. harei* had the lowest resistance rates for cefoxitin, ertapenem and meropenem. The difference in antibiotic resistance shows that it is important to identify the GPAC strains encountered in anaerobic infections and also to perform regular surveillance on the resistance rates of GPAC. Only then the relevance of the individual species with regard to specific sites of infection, ecological changes and development of resistance problems can be studied properly. It is hereby important to properly identify the GPAC strains, using either genotypic methods or MALDI-TOF MS instead of the classical phenotypic method. It is worthwhile to mention that the clinically most encountered GPAC *F. magna* has the highest resistance rates. The features of *F. magna* and its possible role in clinical infections is reviewed in Chapter 8.

The role of F. magna in clinical infections.

This Chapter is based on an extensive study of the literature on *F. magna*. *F. magna* is able to produce several different virulence factors; collagenase, an albumin binding protein (PAB), protein L, a subtilisin-like proteinase (SufA), and an *F.magna* adhesion factor (FAF). The presence of a certain virulence factor is related to the site of infection. *F. magna* strains isolated from intra-abdominal infections have the lowest enzymatic activity, while strains isolated from nonpuerperal breast abscesses and diabetic foot infections have the highest collagenase production [7, 8]. There is a correlation between protein L expressing *F. magna* strains and bacterial vaginosis [2, 6] and *F. magna* strains from suppurative infections prefer to produce PAB [2]. In these studies it is also shown that none of the *F. magna* strains produce protein-L and PAB at the same time. Genome analysis of *F. magna* revealed that it consists of a circular chromosome and a plasmid [3]. The most remarkable feature is the presence of 7 sortase homologs on the plasmid. Sortases are extracellular transpeptidases that catalyze the cell anchoring of cell-wall proteins. The presence of this number of sortases, actually the highest number found in gram-positive bacteria so far, highly contributes to the virulence of *F. magna*. There is no evidence of a concerted action of the various virulence factors. Two studies described [2, 9] that protein L expressing strains do not express PAB. However, the mechanism behind this

feature or whether this applies also for other virulence factors is unknown. Further research on the pathogenicity of *F. magna* is warranted, especially when considering that *F. magna* shows the highest resistance level of all GPAC and that this species is most often encountered in anaerobic infections in which GPAC play a role.

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Nederlandse samenvatting en algemene discussie

Samenvatting en algemene discussie

In dit proefschrift worden de biochemische en moleculaire technieken geëvalueerd en toegepast welke gebruikt kunnen worden voor de identificatie van gram-positieve anaërobe kokken (GPAC) op genus- en speciesniveau. Tijdens deze studies werd een uitgebreide en goed gedefinieerde collectie van GPAC-stammen aangelegd. Hierdoor was het mogelijk om een diepgaande studie naar de antibioticaresistentie van de verschillende GPAC-species te verrichten. Gezien hun klinische relevantie is het belangrijk om goed inzicht te krijgen in de toenemende antibioticaresistentie van anaërobe bacterien. In dit proefschrift wordt de identificatie, klinische relevantie en antibioticaresistentie van GPAC besproken. De klinische relevantie wordt benadrukt door een review over de pathogenese van *Finegoldia magna*.

Fenotypische identificatie gebaseerd op biochemische eigenschappen

In hoofdstuk 2 worden GPAC geïdentificeerd m.b.v. de commercieel verkrijgbare Vitek-ANC-kaart. De identificatie wordt vergeleken met de genotypische identificatie verkregen door de sequentie van het 16S rRNA te bepalen. De database van het Vitek-systeem bevat slechts een gelimiteerd aantal GPAC-species, waaronder de twee meest voorkomende *F. magna* en *Parvimonas* (*Pa.*) *micra*. De derde meest voorkomende species, *Peptoniphilus* (*Pn.*) *harei*, is niet aanwezig in de database en wordt fout geïdentificeerd als *Peptoniphilus asaccharolyticus*. M.b.v. de Vitek-ANC-kaart wordt 51,9 % van de GPAC goed geïdentificeerd op speciesniveau en 69,2 % op genusniveau. In het algemeen vertoond de ANC-kaart dezelfde beperkingen als de klassieke fenotypische identificatiemethoden [5]. Zo kan bv. *Pn. harei* fenotypisch niet onderscheiden worden van *Pn. asaccharolyticus*. *Pn. harei* wordt net zo frequent aangetroffen in klinisch materiaal als *Pa. micra*. Indien de ANC-kaart een isolaat zou identificeren als *Pn. harei* of *Pn. asaccharolyticus*, i.p.v. alleen *Pn. asaccharolyticus*, zou het percentage correcte speciesidentificatie toenemen van 63,5 % naar 78,8 %. Het ware correct als dit identificatiesysteem niet pretendeert op speciesniveau te kunnen determineren, daar species behorend tot het belangrijke genus *Peptoniphilus* slechts op genusniveau geïdentificeerd kunnen worden. Species behorende tot het genus *Anaerococcus* konden niet worden geïdentificeerd of werden fout geïdentificeerd. Uit deze studie is gebleken dat m.b.v. de ANC-kaart de species *F. magna*, *Pa. micra* en *P. anaerobius* betrouwbaar geïdentificeerd kunnen worden, maar andere GPAC-species niet.

In hoofdstuk 3 worden klinisch relevante GPAC fenotypisch geïdentificeerd m.b.v. de voor de identificatie relevante enzymtesten uit het commercieel

verkrijgbare Rapid ID 32A-systeem. M.b.v. de enzymtesten uit dit soort systemen wordt een code verkregen welke ingevoerd kan worden in een database. Hiermee wordt een identificatie verkregen. Dit soort databases bevat echter een gelimiteerd aantal species en zijn vaak niet up to date voor nieuw beschreven species. Zo kan b.v. met het invoeren van de code voor *Peptoniphilus ivorii* de identificatie *Clostridium difficile* worden verkregen. In onze studie werden alleen de voor de identificatie relevante enzymtesten gbruikt en resultaten vergeleken met de genotypische identificatie welke gebaseerd is op het bepalen van de volgorde van het 16S rRNA of fluorescente in situ hybridisatie (FISH) waarbij gebruik is gemaakt van species-specifieke probes. *F. magna* (29/29) en *Pa. micra* (24/28) konden betrouwbaar worden geïdentificeerd met deze fenotypische methode van identificatie. Dit geldt echter niet voor *Pn. harei* (n=17). Ook met deze methode kon deze species niet worden onderscheiden van *Pn. asaccharolyticus*. Tevens werden van de 17 geteste stammen 4 fout of in het geheel niet geïdentificeerd. *Peptostreptococcus anaerobius* en *Peptococcus niger* werden fenotypisch correct geïdentificeerd. *Pn. ivorii* en *Anaerococcus vaginalis* konden betrouwbaar worden geïdentificeerd m.b.v. de relevante enzymtesten, maar werden ook wel eens fout geïdentificeerd. Andere GPAC species zoals *Anaerococcus lactolyticus*, *Peptoniphilus lacrimalis*, *Peptoniphilus octavius* en *Anaerococcus tetradius* konden niet met deze methode worden geïdentificeerd.

Genotypische identificatie m.b.v. op het 16S rRNA gebaseerde probes en analyse van 16S rRNA sequenties.

In hoofdstuk 3 wordt de ontwikkeling van species-specifieke op het 16S rRNA gebaseerde probes beschreven. De sequentieresultaten van het 16S rRNA-gen van klinische isolaten laten voor sommige species een grote intra-species variatie zien. Hierdoor kan het mogelijk zijn dat ontworpen species-specifieke 16S rRNA probes alleen gebaseerd op sequenties van referentiestammen niet met alle stammen van hetzelfde species hybridiseren. Om dit probleem op te lossen zijn de probes niet alleen gebaseerd op sequenties van referentiestammen, maar ook op sequenties van klinische isolaten. Er werden species-specifieke probes ontworpen voor *F. magna*, *Pa. micra*, *Pn. harei*, *A. lactolyticus*, *A. vaginalis*, *Pn. ivorii* en *P. anaerobius/stomatis*. De validatie van deze probes op referentiestammen en klinische isolaten, waarvan de sequenties werden gebruikt om de probes te ontwerpen, liet zien dat ze species-specifiek zijn. De verkregen set probes werd gebruikt om 100 onbekende klinische isolaten te identificeren. Indien isolaten niet konden worden geïdentificeerd met deze set probes, werd de sequentie van het 16S rRNA-gen bepaald. Hiermee werd bepaald of het om een species ging die niet werd gedetecteerd met de set probes, of dat het om een fout-negatief resultaat

ging. Van de 100 isolaten konden 87 geïdentificeerd worden. De sequentieresultaten van de overige 13 stammen lieten zien dat het voornamelijk om species ging welke niet gedetecteerd werden met de set probes. Er waren echter 2 stammen van *A. vaginalis* en 1 stam van *A. lactolyticus* welke negatief waren, doordat ze mismatches hadden met hun corresponderende probe. Hill et al. [4] beschreven voor deze 2 stammen een grote intra-speciesvariatie. Het ontwerpen van species-specifieke probes voor species met zo'n grote intra-speciesvariatie kan resulteren in fout-negatieve resultaten, zoals in onze studie het geval was.

Analyse van de sequentiedata van 71 klinische isolaten, waarvan de volledige lengte van de sequentie werd verkregen, liet zien dat er 25 isolaten zijn met een sequentie-overeenkomst van <98 % met hun meest verwante species. Dit geeft een grote intra-speciesvariatie aan voor sommige species, zoals *A. lactolyticus*, *Pn. harei* en *Pn. ivorii*. Het is echter ook mogelijk dat deze stammen één of meerdere nieuwe species vertegenwoordigen. Uitgebreidere studies zullen moeten worden uitgevoerd om te bepalen of dit werkelijk het geval is. Na de publicatie van de studie beschreven in dit hoofdstuk, werden 3 nieuwe species beschreven door Song et al. [10]. Twee daarvan zijn *Peptoniphilus gorbachii* en *Anaerococcus murdochii*. De meest verwante van deze species zijn respectievelijk *Pn. harei* en *A. lactolyticus*. Sequentieanalyse van stammen van *Pn. harei* (n=4) en *A. lactolyticus* (n=7) met een sequentie-overkomst van <98 % toont aan dat een aantal van deze stammen *Pn. gorbachii* (n=1) en *A. murdochii* (n=5) zijn. Dit houdt in dat de species-specifieke probes voor *Pn. harei* en *A. lactolyticus* niet alleen met de overeenkomende species hybridiseren maar ook met de nieuwe species. Dit is een nadeel van het gebruik van species-specifieke probes voor de identificatie van bacteriën. Probes kunnen hun species-specificiteit verliezen door toevoeging van nieuwe species aan een genus. Het is daarom belangrijk om probes regelmatig te controleren m.b.v. een openbare database zoals Genbank. Een belangrijk voordeel van het gebruik van FISH voor de identificatie van bacteriën is de directe toepasbaarheid op klinisch materiaal.

In hoofdstuk 4 wordt een patiëntencasus omschreven van een tongabces. Deze casus laat duidelijk zien hoe FISH een belangrijke rol heeft gespeeld bij de identificatie van de verschillende species die aanwezig waren in het van het abces afkomstige purulente klinische materiaal. Species welke gedetecteerd werden m.b.v. FISH konden niet uit het materiaal worden geïsoleerd door gebruik te maken van de klassieke kweekmethoden.

Door 100 onbekende klinische isolaten van GPAC te identificeren m.b.v. moleculaire technieken, werd er een beter inzicht verkregen in de klinische relevantie van een aantal van deze species. De meest geïsoleerde species uit

klinisch materiaal zijn *F. magna* en *Pa. micra*. Dit werd bevestigd in onze studie. Echter ook *Pn. asaccharolyticus* wordt beschreven als veel voorkomend. In onze studie werd geen *Pn. asaccharolyticus* aangetroffen, maar wel waren 17 % van alle GPAC isolaten positief met de species-specifieke *Pn. harei* probe. Song et al. [11] gebruikten een multiplex-PCR-assay met genus- en species-specifieke primers voor de identificatie van GPAC. Met deze assay werd in totaal 190 GPAC-stammen geïdentificeerd, waarvan 25 % als *Pn. harei*. Evenals in onze studie werden geen *Pn. asaccharolyticus*-stammen aangetroffen. De biochemische eigenschappen van *Pn. harei* en *Pn. asaccharolyticus* laten zien dat deze 2 species niet fenotypisch van elkaar onderscheiden kunnen worden [5]. Uit de resultaten blijkt duidelijk dat in het verleden *Pn. harei* fout geïdentificeerd is als *Pn. asaccharolyticus*. Zoals beschreven in hoofdstuk 5, wordt de klinische relevantie van *Pn. asaccharolyticus* duidelijk overschat en *Pn. harei* wordt vaker geïsoleerd uit klinisch materiaal dan eerder werd aangenomen.

Identificatie m.b.v. MALDI-TOF MS

Recentelijk is de populariteit van een andere fenotypische methode voor de identificatie van bacteriën aanzienlijk toegenomen, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). In hoofdstuk 6 wordt beschreven hoe deze techniek wordt gebruikt voor de identificatie van GPAC. Een database werd samengesteld door gebruik te maken van klinische isolaten waarvan de sequentie van het 16S rRNA is bepaald en referentiestammen. De verkregen database werd gebruikt voor de identificatie van 107 onbekende klinische isolaten. De MALDI-TOF MS-identificatie werd vergeleken met de genotypische identificatie, hetzij door de sequentie van het 16S rRNA te bepalen of m.b.v. FISH door gebruik te maken van de species-specifieke probes beschreven in hoofdstuk 3. Van deze 107 isolaten konden er 11 niet geïdentificeerd worden, waarvan 5 omdat er geen referentiespectra aanwezig waren in de database. De overige 6 isolaten werden genotypisch geïdentificeerd als *F. magna* (n=3), *A. vaginalis* (n=1) en *Pn. ivorii* (n=2). Voor 5 van deze stammen kon de sequentie overeenkomst met hun meest verwante worden berekend en 3 van de stammen hadden een sequentie overeenkomst van <98 % (1 *F. magna* isolaat en 3 *Pn. ivorii* isolaten). Het met MALDI-TOF MS verkregen dendrogram liet zien dat alle geanalyseerde klinische isolaten samen klusteren met hun meest verwante, behalve de 6 stammen welke niet geïdentificeerd konden worden ondanks de aanwezigheid van referentiespectra in de database. Wanneer het dendrogram wordt vergeleken met de fylogenetische boom verkregen m.b.v. de 16S rRNA-sequenties, worden dezelfde verwantschappen tussen de verschillende stammen waargenomen. Dit komt waarschijnlijk door het feit dat met MALDI-TOF

MS, naast andere eiwitten, voornamelijk ribosomale eiwitten worden gemeten [1]. De resultaten van deze studie laten zien dat met MALDI-TOF MS niet alleen betrouwbaar GPAC-isolaten kunnen worden geïdentificeerd, maar dat het ook geschikt is voor het beschrijven van de taxonomie tussen de verschillende species. De analyse van de 16S rRNA-sequenties van de GPAC isolaten en de MALDI-TOF MS-resultaten laten beide nieuwe nog niet beschreven species zien.

De antibioticagevoeligheid van GPAC

In hoofdstuk 7 wordt de antibioticagevoeligheid tussen de verschillende GPAC-species beschreven. Vijftien verschillende GPAC-species werden getest met 14 verschillende antibiotica. In tegenstelling tot andere studies, werden de isolaten genotypisch geïdentificeerd m.b.v. FISH of door de sequentie van het 16S rRNA te bepalen. Hierdoor is dit de eerste antibioticaresistentiestudie waarin *Pn. harei* betrouwbaar geïdentificeerd is, en zijn antibioticagevoeligheid vergeleken kan worden met andere GPAC. Tevens worden in deze studie de eerste antibioticaresistentiedata gegeven voor de nieuwe species *Pn. gorbachii* en *A. murdochii*. Vaak wordt in studies de resistentiedata gepresenteeerd als het percentage resistente stammen. Hierbij worden echter verschillende breekpunten voor hetzelfde antibioticum gebruikt, met name volgens de interpretatieve kriteria van CLSI en EUCAST. Dit maakt het moeilijk om de data van de verschillende studies met elkaar te vergelijken. Om deze reden hebben wij er voor gekozen om onze resistentiedata te bespreken aan de hand van de MIC_{50} en MIC_{90}, voor de verschillende antibiotica voor elk species. Deze waarden geven aan bij welke concentratie antibiotica 50% respectievelijk 90% van de stammen geremd worden in hun groei. De MIC_{50} en MIC_{90} werd alleen berekend als er van één species meer dan 10 isolaten waren. In feite waren dit de 3 meest voorkomende GPAC-species *F. magna*, *Pa. micra* en *Pn. harei*. In het algemeen wordt de hoogste resistentie (MIC_{50} en MIC_{90}) aangetroffen bij *F. magna* voor de antibiotica penicilline G, amoxicilline-clavulaanzuur, clindamycine en tigecycline. *Pa. micra* had de laagste resistentie voor levofloxacine, metronidazol en doxycycline. *Pn. harei* had de laagste resistentie voor cefoxitin, ertapenem en meropenem. Het verschil in antibioticaresistentie geeft aan dat het belangrijk is om GPAC-isolaten, aangetroffen in anaërobe infecties, te identificeren en tevens een reguliere surveillance uit te voeren op het voorkomen van antibioticaresistentie van GPAC. Alleen dan kan de relevantie van de individuele species m.b.t. een specifieke plaats van infectie, ecologische veranderingen en de ontwikkeling van resistentieproblemen grondig worden bestudeerd. Hierbij is het belangrijk om de GPAC-stammen goed te identificeren, door gebruik te maken van hetzij genotypische methoden of MALDI-TOF MS i.p.v. klassieke fenotypische methoden.

Het is de moeite waard om te vermelden dat de klinisch meest voorkomende GPAC *F. magna* over het algemeen de hoogste MIC_{50} en MIC_{90} voor antibiotica heeft. De eigenschappen van *F. magna* en zijn mogelijke rol in klinische infecties wordt besproken in hoofdstuk 8.

De rol van F. magna in klinische infecties

Dit hoofdstuk is gebaseerd op een studie van de literatuur over *F. magna*. *F. magna* is in staat om verschillende virulentiefactoren te produceren; collagenase, een albumine-bindend eiwit (PAB), protein L, een op subtilisine gelijkend proteinase (SufA), en een *F. magna* adhesiefactor (FAF). De aanwezigheid van een bepaald virulentiefactor is gerelateerd aan een ziekteproces waarmee het isolaat is geassocieerd. *F. magna* stammen geïsoleerd uit intra-abdominale infecties hebben de laagste enzymatische activiteit, terwijl stammen geïsoleerd uit niet-zwangerschap gerelateerde borstabcessen en diabetische voet infecties de hoogste collagenaseproduktie hebben [7, 8]. Er is een correlatie tussen *F. magna*-stammen die protein L tot expressie brengen en bacteriële vaginose [2, 6], terwijl *F. magna*-stammen geïsoleerd uit suppuratieve infecties bij voorkeur PAB produceren [2]. Uit deze studies is tevens gebleken dat geen van de *F. magna*-stammen in staat is om tegelijkertijd protein L en PAB te produceren. Uit de genoomanalyse van *F. magna* blijkt dat het bestaat uit een circulair chromosoom en een plasmide [3]. Het meest opvallende kenmerk is de aanwezigheid van 7 sortase-homologen op het plasmide. Sortases zijn extracellulaire transpeptidases die de verankering van celwand gebonden eiwitten katalyseren. De aanwezigheid van dit aantal sortases, het hoogste aantal tot nu toe aangetroffen in gram-positieve bacteriën, draagt in hoge mate bij aan de virulentie van *F. magna*. Tot nu toe is er geen bewijs dat de verschillende virulentiefactoren gezamenlijk kunnen handelen, waarbij ze eventueel elkaars virulentie zouden kunnen versterken. Twee studies beschrijven [2, 9] dat stammen die protein L tot expressie brengen geen PAB tot expressie brengen. Het mechanisme achter deze eigenschap en of dat dit ook van toepassing is voor andere virulentiefactoren is onbekend. Verder onderzoek naar de pathogenese van *F. magna* is van belang, met name ook omdat *F. magna* de hoogste resistentie voor antibiotica heeft van alle GPAC-species en het meest voorkomt in anaërobe infecties waarin GPAC een rol spelen.

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List of abbrevations

List of abbrevations

ANC	anaerobic and Corynebacteria identification card
ATCC	American type culture collection
BA	blood agar
BAZ	blood aztreonam agar
BBA	brucella blood agar
BBE	Bacteroides bile esculin agar
CCUG	culture collection of the University of Göteburg
CHOC	chocolate agar
CLSI	clinical and laboratory standards institute
COB	colistin blood agar
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSM	deutsche sammlung von mikroorganismen
EDTA	ethylenediaminetetra acetic acid
EMBL	european molecular biology laboratory
EUCAST	european committee on antimicrobial susceptibility testing
FAF	*F. magna* adhesion factor
FISH	fluorescent in situ hybridisation
FITC	fluorescein-5-isothiocyanate
FPA/FPB	fibrinopeptides
GA	protein G-related albumin binding
GLC	gas liquid chromatography
GPAC	gram-positive anaerobic cocci
HSA	human serum albumin
Ig	immunoglobulin
KCl	potassium chloride
KVLB	kanamycin-vancomycin blood agar
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MC	McConkey agar
$MgCl_2$	magnesium chloride
MIC	minimal inhibitory concentration
MIS	microbial identification system
MMBA	mupirocin-metronidazole blood agar
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NH_2-	amino-

PAB	peptostreptococcal albumin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEA	phenylethyl alcohol blood agar
PMS	pyrolysis mass spectrometry
PTS	phosphotransferase system
RDP	ribosomal database project
rRNA	ribosomal ribonucleic acid
SAB	Sabouraud dextrose agar
SDS	sodium dodecyl sulphate
SPS	sodium polyanethol sulfonate
SufA	subtisilin-like proteinase
VFA	volatile fatty acids

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List of publications

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2. EHL Lee, JE Degener, GW Welling, **ACM Veloo.** Evalution of the VITEK 2 ANC Card for the identification of clinical isolates of anaerobic bacteria. J. Clin. Microbiol. 2011; 49:1745-1749.

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Dankwoord

En dan is het opeens af, het proefschrift, en moet alleen nog het moeilijkste en meestgelezen hoofdstuk worden geschreven, het dankwoord.

John, bedankt voor de kans om als analist een promotie-onderzoek uit te mogen voeren. Als mijn promotor heb je me altijd bijgestaan en gestimuleerd. Voor vragen kon rustig mijn hoofd om de deur steken om ze even te stellen. Bedankt voor al je steun, geduld en begrip. De discussies over de lopende onderzoeken wierpen vaak weer een nieuw licht op zaken, waardoor ik weer verder kon.

Gjalt, je hebt me als beginnend analist meegemaakt en samen hebben we, naast de anaëroben, ook de nodige darm-projecten afgerond. Ook jij was er altijd om vragen te beantwoorden en de nodige correcties aan te brengen in de stukken die ik geschreven had. Ongemerkt heb je me de nodige stimulans gegeven om dit proefschrift af te ronden.

De anaërobe werkgroep. Zonder jullie was het niet mogelijk geweest om de onderzoeken uit te voeren. Bedankt voor het verzamelen van de nodige stammen en het feit dat jullie anaëroben net zo als ik ook leuk vinden.

De PCR-groep. Jullie hebben heel wat stammen van mij te verwerken gekregen en het kon altijd.

Al mijn naaste collega's. Hermie, bedankt voor al je raad als het ging om sequentie analyses en het maken van fylogenetische boompjes. Rudi, voor al onze gezellige gesprekken en je steun bij de nodige computerproblemen. Erwin en Carien, voor jullie adviezen en kletspraat op de kamer. Gerr, voor al onze gezellige rookmomentjes en je goede raad. Tanweer, Medhi, Sandra, Marcus en oud collega's, die ik niet bij naam zal noemen uit vrees dat ik er eentje vergeet, bedankt voor alle wetenschappelijke discussies.

Pap en mam, bedankt voor jullie steun en trots. Jullie zijn er altijd voor me, ook in moeilijke tijden.

Mathijs, mijn lieve kereltje. Jouw heerlijke fantasie en liefde voor mama maakt elke dag weer goed.